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(54) Title: RECOMBINANT THYROTROPIN RECEPTOR

(57) Abstract

Functionally-active, human thyrotropin receptor has been expressed in non-thyroidal eukaryotic cells. A human thyroid cDNA library was screened with two synthetic oligonucleotides based on the reported amino acid sequence of the 3rd and 4th transmembrane domains of a putative human thyrotropin receptor and related receptors. The nucleotide sequence of a 4 kb clone revealed an open reading frame of 764 amino acids (86,816 Daltons) with a putative signal peptide, seven transmembrane domains, five potential glycosylation sites, and a very short intracytoplasmic region. Homology with the extracellular domain of the pig LH/CG receptor was only 33 %. Chinese hamster ovary cells stably transfected with this cDNA in an expression vector generated a functional receptor, able to activate adenylate cyclase, specifically in response to thyrotropin stimulation.

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TITLE OF THE INVENTION

RECOMBINANT THYROTROPIN RECEPTOR

Cross-Reference to Related Applications

This is a continuation-in-part of U.S. Application Serial No. 07/575,018, filed August 30, 1990, which is a continuation-in-part of U.S. Application Serial No. 07/451,973, filed December 20, 1989.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of molecular biology and immunology. More particularly, the invention relates to the production of recombinant thyrotropin receptor in non-thyroidal eukaryotic cells. The invention is further related to methods of using recombinant thyrotropin receptor, and, in particular, to methods of using recombinant thyrotropin receptor in diagnosis and differentiation between autoimmune and non-autoimmune causes of thyrotoxicosis. The invention is also related to the treatment of immune disorders such as Graves' Disease.

Brief Description of the Related Art

The most important of the diseases that cause thyrotoxicosis is Graves' disease, also known as Parry's or Basedow's disease. However, not all hyperthyroidism is a result of Graves' disease. Additionally, not all thyrotoxicosis is due to hyperthyroidism. Thyrotoxicosis is the clinical, biochemical and physiological result of sustained delivery of excessive quantities of thyroid hormones to the peripheral tissues. Hyperthyroidism is used to denote the situation where this excess of hormone is the result of sustained thyroid hyperfunction.

There are a number of causes for thyrotoxicosis, the major ones include: Graves' disease, toxic adenoma, toxic multinodular goiter, thyrotoxicosis factitia, ectopic thyroid, and thyroiditis. A portion

of these are hyperthyroid in nature whereas the remaining portion is nonhyperthyroid. Graves' disease is by far the most common cause of thyrotoxicosis, as well as the only autoimmune cause. It is therefore of extreme importance that an accurate assay be available to allow the differentiation between the autoimmune and non-autoimmune varieties; since the treatments, and pathogenesis differ so drastically. Bardin, C.W., Current Therapy in Endocrinology and Metabolism-3, B.C. Decker Inc. Toronto (1988).

Graves' disease is a relatively common disorder that occurs at any age, but most often in the third and fourth decades. The disease is more frequent in women and the ratio of predominance in women may be as high as 7:1. The manifestations of Graves' disease include one or more of the following: hyperthyroidism with diffuse goiter; ophthalmopathy; and dermopathy.

Thyrotropin, also known as thyroid stimulating hormone (TSH), is the primary hormone that regulates thyroid cell differentiated function and proliferation (Dumont, J.E., et al., Adv. Cyclic Nucleotide Res. 14:479-489 (1981)). These effects are mediated by its interaction with the thyrotropin (TSH) receptor on the plasma membrane of thyroid cells. Thyroid stimulating auto-antibodies, the cause of thyrotoxicosis in Graves' disease, mimic the actions of TSH by their interaction with the TSH receptor (Rees Smith, B., et al., Endocr. Rev. 9:106-121 (1988)). In Graves' disease, anti-TSH receptor auto-antibody binds to the TSH receptor on the thyroid cell surface. Such binding causes unregulated stimulation of the thyroid cell which then produces excessive amounts of thyroid hormone.

Because of its pivotal role in the pathophysiology of the thyroid cell, the molecular cloning and expression of the TSH receptor has been a long-standing, but elusive, goal for many laboratories. There has been no molecular characterization of the TSH receptor. Previous studies have not even agreed as to the size of the TSH receptor. Conventional approaches for the molecular cloning of the TSH receptor have been thwarted by the inability to purify this molecule, primarily because of its extraordinary low abundance on thyroid cells. Attempts to use polyclonal or monoclonal antibodies against the TSH receptor (Yoshida, T., et al., Clin. Res. 36:610A (Abstract) (1988); Chan, J.Y.C., et al., J. Biol. Chem. 264:3651-3654 (1989); H. Hirayu and B. Rapoport, unpublished data), as well as a low-stringency polymerase chain reaction (PCR) approach (Libert, F., et al., Science 244:569-572 (1989)), have also been unsuccessful.

Crude extracts of pig thyroid membranes are currently being used in assays to detect anti-TSH receptor antibodies in the sera of patients with autoimmune thyroid diseases, in particular Graves' disease. This assay, manufactured by R.S.R. Ltd., Cardiff, Wales, is of limited value because it does not distinguish between stimulatory and non-stimulatory (or even inhibitory) anti-TSH receptor antibodies. Additionally, the sensitivity of this assay is insufficient to detect antibodies in all patients with active Graves' disease.

There are functional bioassays for detecting human TSH receptor antibodies. However, these assays are cumbersome and expensive, and generally involve the measurement of the cyclic AMP response to antibody

stimulation in cultured human or rat thyroid cells. These assays are more specific than TSH receptor binding assays, but are less practical.

The LH\CG receptor (McFarland, K.C., et al., Science 245:494-499 (1989); Loosfelt, H., et al., Science 245:525-528 (1989)), a more abundant member of the same glycoprotein hormone receptor family as the TSH receptor, has now been molecularly cloned and facilitated the molecular cloning of the TSH receptor. Frazier-Seabrook et al. (Frazier-Seabrook, L., et al., 64th Meeting of American Thyroid Association T-51 (abstract) (1989)) have presented preliminary data on the isolation, without functional expression, of a human TSH receptor cDNA clone.

At present, there is no therapy that can cure Graves' disease. There are several current treatments with many drawbacks. In one treatment for Graves' disease, drugs are administered that block thyroid hormone synthesis. These drugs are administered for many months or years, while waiting for a spontaneous remission of the thyroid overactivity. Another radical treatment requires ablation of part or all of the thyroid by surgery or radioactive iodine. This commonly leads to hypothyroidism and the need for life-long administration of thyroid hormone.

While there are some treatments available, there are none available which are directed primarily at the underlying immunologic cause of Graves' disease. Therefore, on the whole, the anti-thyroid agents have a low frequency of producing a longterm or permanent remission of the disease. Bardin, supra. The current invention, however, is directed towards treating Graves' disease immunologically.

SUMMARY OF THE INVENTION

In order to obtain full-length TSH receptor free of other potential thyroid antigens for studying the pathogenesis of Graves' disease, the present inventors therefore attempted, and herein report, the expression of recombinant human TSH receptor in non-thyroidal eukaryotic cells. Like native human TSH receptor, this recombinant TSH receptor is functionally active and is not a fusion protein.

It is an object of the present invention, then, to provide for a convenient and economical source of recombinant TSH receptor. The present invention thus provides a number of important advances in the characterization of the TSH receptor. Recombinant, functionally active TSH receptor has been generated in non-thyroidal eukaryotic cells.

With seven transmembrane domains, the TSH receptor belongs to a family of G protein-coupled receptors, including the receptors for LH\CG, substance K, rhodopsin, serotonin, as well as the α1-, α2-, β1- and β2-adrenergic and muscarinic cholinergic receptors (McFarland, K.C., et al., Science 245:494-499 (1989); Loosfelt, H., et al., Science 245:525-528 (1989)). In contrast to the short extracellular domains recognized by the smaller ligands such as adrenergic and cholinergic agents, the extracellular domain of the TSH receptor is much larger and more complex. This finding is consistent with the complexity of the glycoprotein hormones, which are approximately 30 kD in size, and suggests that the extracellular domain plays an important role in hormone binding and signal transduction. Of interest

is a 50 amino acid insertion upstream of the transmembrane domain that is unique to the TSH receptor, and which is therefore a potential TSH binding domain.

The molecular cloning of the TSH receptor now opens the way for future studies to answer many questions that have remained unanswered for a number of years. These questions include a) the site(s) of binding (epitopes) of stimulatory and inhibitory anti-TSH receptor antibodies present in the sera of patients with autoimmune thyroid disease, and the relationship between these binding sites and that for TSH; b) determination of the mechanism of signal transduction by which TSH increases adenylate cyclase activity in thyroid cells; and c) the mechanism by which continued TSH stimulation leads to a decrease in TSH receptor-coupling with G_s and reduced adenylate cyclase activation (homologous desensitization).

The production of recombinant TSH receptor also makes possible new treatments for thyrotoxicosis which would not have the drawbacks of current therapies.

Thus, in one embodiment, there is provided according to the invention recombinant, functionally active thyrotropin receptor, or a functional or chemical derivative thereof.

In another embodiment is provided thyrotropin receptor which is produced by non-thyroidal eukaryotic cells. Yet another embodiment of the invention comprises the plasmid pSV2-NEO-ECE-hTSHR. There is also provided according to the invention a non-thyroidal eukaryotic cell transformed with this plasmid, as well as methods of producing thyrotropin receptor, comprising culturing the transformed cell

under conditions allowing expression of thyrotropin receptor, and recovering said thyrotropin receptor.

In yet another embodiment, the present invention provides for an antibody against the thyrotropin receptor of the invention.

Further, a method of detecting thyrotropin receptor in a sample is provided according to the present invention, comprising contacting said sample with an antibody crossreactive with the thytropin receptor, wherein said antibody is detectably labeled, so as to form a complex between the thyrotropin receptor in said sample and said detectably labeled antibody, and detecting the complexed or uncomplexed detectably labeled antibody. In an additional embodiment, there is provided a kit for the detection of thyrotropin receptor in a sample, comprising container means comprising one or more containers, wherein one of said containers comprises detectably labeled antibody against thyrotropin receptor.

Moreover, a method of detecting antibodies to thyrotropin receptor in a sample is provided according to the present invention, comprising contacting said sample with the recombinant thyrotropin receptor, wherein said thyrotropin receptor is detectably labeled, so as to form a complex between the TSH receptor antibodies in said sample and said detectably labeled recombinant TSH receptor, and detecting the complexed antibody. In an additional embodiment, there is provided a kit for the detection of antibodies to thyrotropin receptor in a sample, comprising container means comprising one or more containers, wherein one of said containers comprises detectably labeled recombinant TSH receptor.

An additional embodiment of the current invention comprises a method for differentiating between autoimmune and non-autoimmune varieties of thyrotoxicosis comprising an assay specific for either thyrotropin receptor, anti-thyrotropin receptor autoantibodies or functional or chemical derivatives thereof.

Another embodiment of the current invention comprises a method of treating thyrotoxicosis with pharmacologically effective amounts of recombinant thyrotropin receptor, or a functional or chemical derivative thereof.

In an additional embodiment, a thymus-derived lymphocyte (T cell) is provided which is specific for the autoimmune T-cell receptor (TCR) for TSH receptor. Another embodiment of the invention provides for a pharmaceutical preparation comprising a T cell which is specific for the autoimmune TCR for TSH receptor. In yet another embodiment of the current invention, a method of treating autoimmune thyrotoxicosis is provided comprising the use of a pharmaceutical preparation comprising a T cell which is specific for the autoimmune TCR for TSH receptor.

Another embodiment provides a peptide which is specific for the autoimmune TCR for TSH receptor. Another embodiment of the invention provides for a pharmaceutical preparation comprising a peptide which is specific for the autoimmune TCR for TSH receptor. In yet another embodiment of the current invention, a method of treating autoimmune thyrotoxicosis is provided comprising the use of a pharmaceutical preparation comprising a peptide which is specific for the autoimmune TCR for TSH receptor.

In an additional embodiment, there is provided suppressor T cells specific for anti-thyrotropin receptor auto-antibodies. Another embodiment provides a pharmaceutical preparation comprising suppressor T cells specific for anti-thyrotropin receptor auto-antibodies. Yet another embodiment provides a method of treating thyrotoxicosis with a pharmaceutical preparation comprising T cells specific for anti-thyrotropin receptor auto-antibodies.

These and other non-limiting embodiments of the present invention will be apparent to those of skill from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Nucleotide and derived amino acid sequence of the cDNA for clone 4, the human TSH receptor. Amino acids are annotated by the single letter amino acid code. Potential glycosylation sites are underlined.

Figure 2: Hydropathy plot (17) of the translated region of the TSH receptor. A putative signal peptide is present at the amino terminus. Seven potential transmembrane domains are marked by the horizontal bars.

Figure 3: Comparison of the amino acid sequences of the human TSH receptor and the pig LH\CG receptor. --- - aligned identical residues; upper case - aligned non-identical residues; lower case - unaligned residues; .... - gaps.

Figure 4: Functional activity of the human TSH receptor stably transfected into Chinese hamster ovary (CHO) cells. Cells were exposed for 1 hour to the indicated hormones prior to the measurement of intracellular cAMP levels. 10 mU/ml TSH is equivalent to 10^{-8} M. Brackets indicate the range of determinations in duplicate dishes of cells. The horizontal line indicates the lack of effect of 10 mU/ml TSH on untransfected, wild-type (CHO-K1) cell cAMP levels, and a similar lack of effect of 10^{-7} M hCG, (1-24)ACTH and insulin on cAMP levels on cells transfected with pSV2-NEO-ECE-hTSHR.

Figure 5: Schematic representation of the extracellular domains of the mutant human TSH receptors. The solid bars represent segments in the TSH receptor that are not present in the LH receptor. Lines interrupting the open bars represent deletions in the TSH receptor. The hatched bar indicates substitution rather than deletion of 8 amino acids in the TSH receptor. The amino acids are shown in the single letter code. Numbers indicate amino acid positions in the wild-type human TSH receptor, as previously reported (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989)).

Figure 6: Measurement of the intracellular cAMP response to TSI stimulation in pooled clones of stably-transfected CH0 cells. WT-hTSHR -wild type human TSH receptor: The structures of hTSHR-D1-3 and hTSHR-S1 are shown in Figure 1. Each bar (with the exception of hTSHR-S1 which was tested once in duplicate) represents the mean $\pm$ S.E. of values obtained in duplicate dishes in 3 separate experiments using IgG prepared (Kasagi et al., J. Clin. Endocrinol. Metab. 62:855 (1986)) from pools of 4 individual TSI-positive

sera or TSI-negative sera. TSI stimulation was for 2 hr at 37°C. Intracellular cAMP levels were measured as previously described (Hirayu et al., Molec. Cell. Endocrinol. 42:21 (1985)).

Figure 7: Sites of potential glycosylation in the human TSH receptor. All sites are in the extracellular domain of the receptor. Numbers indicate amino acid positions in the wild-type human TSH receptor, as previously reported (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)). 1B: Mutant TSH receptor cDNA plasmid constructs used in these experiments.

Figure 8: Specific TSH binding to recombinant wild-type and mutant TSH receptor forms stably expressed in Chinese hamster ovary (CHO) cells. The binding of ^{125}I-TSH was measured in the presence of increasing concentrations of bTSH, as described in Methods. Non-specific TSH binding was determined in the presence of 10^{-6}M unlabeled TSH, and was subtracted from each point to give the indicated values (<10% of total binding). Each point represents the mean of two duplicate determinations; the data shown are representative of three to five separate experiments.

Figure 9: Cyclic AMP response to TSH stimulation in CHO cells expressing the wild-type and mutated TSH receptors. Incubations and cAMP assay were performed as described in Methods. Data are expressed as the percentage of values obtained in cells incubated under identical conditions in the absence of TSH and are representative of three separate experiments; each point represents the mean of duplicate values determined in duplicate dishes of cells.

Figure 10: Schematic representation of chimeric TSH-LH/CG receptor extracellular domains. The 418

amino acid extracellular region of the human TSH receptor (764 amino acids) was divided into 5 arbitrary domains (A-E) on the basis of the indicated restriction sites. In the chimeras, the open areas denote the region(s) of the extracellular domain of the human TSH receptor that remained after substitution. The solid areas depict the homologous regions of the rat LH/CG receptor that were used to substitute for the deleted TSH receptor domains. The LH/CG receptor fragment inserted into TSH receptor domain D is 50 amino acids smaller than its TSH receptor counterpart, corresponding to the nature of the normal LH/CG receptor in this region (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)), as shown by the horizontal line. Chimeric receptors are designated as TSH-LHR-1 through TSH-LHR-10. The numbers assigned to the amino acids are those published for the human TSH receptor (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)) and for the rat LH/CG receptor (McFarland, K.C., et al., Science 245:494-499 (1989)). Signal peptide sequences have not been deleted. The identity of the LH/CG receptor fragments inserted into the indicated restriction sites in the TSH receptor are indicated.

Figure 11: Desensitization of the cAMP response to TSH stimulation occurs in human thyroid cells, but not in Chinese hamster ovary (CHO) cells expressing recombinant, functional human TSH receptors. Cells were cultured in 35 diameter culture dishes in medium lacking TSH (Methods). In the case of the human thyroid cells, the cells were established in primary culture 4 d previously, also in the absence of TSH. TSH (10 mU/ml) was then added to the medium of some

dishes of cells and the incubation continued for 16 h. The medium was then replaced with fresh medium with or without 10 mU/ml TSH and 2 isobutyl methylxanthine (IBMX). After incubation for 1 h, cellular cAMP was extracted and measured by radioimmunoassay (Methods). Each bar represents the mean ± the range of duplicate values obtained in duplicate dishes. The data are representative of 3 experiments with the CHO-TSHR cells, and more than 100 experiments with the human thyroid cells. Con: control incubations without TSH.

Figure 12: Time course of specific ^{125}I-TSH binding to CHO-TSHR cells expressing recombinant human TSH receptor. In this and subsequent experiments, cells were plated in 24 well Costar plates, and allowed to grow to total confluence. ^{125}I-TSH binding was performed in 0.5 ml of modified Hank's buffer (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)) (Methods). Non-specific binding (approximately 10% of specific binding) was determined in the presence of excess (10^{-6}M) TSH, and this value subtracted from total binding to yield specific binding values. The data are expressed as the percentage of total ^{125}I-TSH added to each well. Similar data were obtained In a separate experiment.

Figure 13: Competition-inhibition by TSH of ^{125}I-TSH binding to CHO cells expressing recombinant human TSH receptors (CHO-TSHR). CHO-TSHR and wild-type CHO cells in 24 well Costar plates were grown to total confluence. ^{125}I-TCH binding was performed for 2 h at 37°C in 0.5 ml of modified Hank's buffer (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)) (Methods) supplemented with the indicated concentration of stable TSH. Nonspecific binding (approximately 15% of specific binding in the

experiment shown) was determined in the presence of excess (10^{-6}M) TSH, and this value subtracted from total counts bound to yield specific binding values. The data are expressed as the percentage of total ^{125}I-TSH added to each well. Each point represents the mean ± range of duplicate determinations, and the experiment shown is representative of three experiments.

Figure 14: Scatchard analysis of specific TSH binding to CHO-TSHR cells. Calculations were performed on the data shown in Fig. 3, and are representative of 3 separate experiments.

Figure 15: The recombinant human TSH receptor in CHO-TSHR cells does not "down-regulate" following prolonged exposure to high concentrations of TSH or dBcAMP. Cells were preincubated for the indicated periods of time in medium containing 10 mU/ml TSH or 1mM dBcAMP. In order to remove non-labeled TSH prior to ^{125}I-TSH binding, the cells were rinsed three times (Methods). Data are expressed as the % of ^{125}I-TSH specifically bound. Absolute specific binding in the experiment shown was 9%. Each point represents the mean ± range of duplicate determinations. Similar data were obtained in two other experiments, one performed at the same TSH concentration (10 mU/ml), and the other at the extremely high level of 1000 mU/ml (10^{-6} M).

Figure 16A: Mutations in the first cytoplasmic loop of the human TSH receptor. In this and subsequent figures amino acid substitutions in the sequence of the wild-type TSH receptor (WT-TSH-R) are boxed. Amino acids are shown in the single letter code. The amino acid numbers shown correspond to those previously reported for the human TSH receptor

(Nagayama, Y. et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)). All the oligonucleotides shown below are in the reverse-complemented orientation, as required for mutagenesis. The sequence of the oligonucleotide used for this mutation was 5' - ACATGAGAAATTGGGGGACGTTCAGTTGGGCTTGGCCGGCGAGGAGAAT.

Figure 16B: TSH binding to CHO cells expressing the wild-type human TSH receptor and TSH receptor mutant MUT1-TSH-R. In this and subsequent figures competition for ^{125}I-TSH binding was performed with the indicated concentrations of unlabeled hormone as described in Methods. Non-specific binding in the presence of 10^{-6}M TSH (5% of total counts bound in the representative experiment shown) was subtracted from total counts bound to yield specific TSH binding. For comparison among clones, maximum specific binding of ^{125}I-TSH in the absence of unlabeled hormone is designated as 100%. Each point represents the mean of two closely agreeing duplicate determinations. The experiments shown are representative of 2 or more different experiments, each made with pooled clones from separate stable transfections. The Kd values for this and other mutants are listed in Table I.

Figure 16C: Cyclic AMP response to TSH stimulation in CHO cells expressing the wild' type human TSH receptor (WT-TSH-R) or the TSH receptor mutated in the first cytoplasmic loop (MUT1-TSH-R). In this and subsequent figures cells were exposed for 1 hour at 37°C to the indicated concentrations of TSH. Data are expressed as the % of values obtained in cells incubated under identical conditions in the absence of TSH. Each point represents the mean of duplicate values determined in duplicate dishes of cells. Similar data were obtained in two or more

experiments, each made with pooled clones from separate transfections.

Figure 17A: Mutations in the second cytoplasmic loop of the human TSH receptor. Substitutions in the sequence of the wild-type TSH receptor (WT-TSH-R) are boxed. Amino acids are shown in the single letter code. The sequences of the oligonucleotides used in these mutations were 5'-GATGGCACATGCGCCCTGGAGGCCGATC-TGCTGGCCCAGTTGCATGGCGAAGGT (MUT2-TSH-R) and 5'-GGCGAAGGCGATGGCAGCCCAGCCCTGCAGGGTGAT (MUT3-TSH-R).

Figure 17B: TSH binding to CHC cells expressing the wild-type human TSH receptor (WT-TSH-R), TSH receptor mutants MUT2-TSH-R and MUT3-TSH-R, and cells transfected with vector not containing the TSH receptor cDNA (pSV2-neo). For details, see the legend to Fig. 1B.

Figure 17C: Cyclic AMP response to TSH stimulation in CHO cells expressing the wild-type human TSH receptor (WT-TSH-R) or the TSH receptor mutated in the second cytoplasmic loop. For details see the legend to Figure 1C.

Figure 18A: Mutations in the third cytoplasmic loop of the human TSH receptor. Substitutions in the sequence of the wild-type TSH receptor (WT-TSH-R) are boxed. Amino acids are shown in the single letter code. The sequences of the oligonucleotides used in these mutations were 5'AGCCATCCCCTGGGCAATTCCGGCATTT-TGGTTCCCTGGGTT (MUT4-TSH-R) and 5'-CCTGGGTTGGCCTGGGGATTTCCGACTGCGATGGGGATCCCCACATGACA (MUT5-TSH-R).

Figure 18B: TSN binding to CHO cells expressing the wild-type human TSH receptor (WT-TSH-R), TSH receptor mutants MUT4-TSH-R and MUT5-TSH-R, and cells transfected with vector not containing the TSH

receptor cDNA (pSV2-neo). For details, see the legend to Fig. 1B.

Figure 18C: Cyclic AMP response to TSH stimulation in CHO cells expressing the wild-type human TSH receptor (WT-TSH-R) or the TSH receptor mutated in the third cytoplasmic loop. For details see the legend to Figure 1C.

Figure 19A: Mutations in the cytoplasmic tail of the human TSH receptor. Substitutions in the sequence of the wild-type TSH receptor (WT-TSH-R) are boxed. Amino acids are shown in the single letter code. The sequences of the oligonucleotides used in these mutations were 5′-ATCCCTCTAGAAGGCCTAGGTGAAAA (MUT6-TSH-R; stop codons at nucleotides 2146 and 2155, shown underlined in the reverse-complemented orientation), 5′-CTCTGCCCCTGGAATGCCTGAGCCTGGCCTTGGCCGATGCCAAA (MUT7-TSH-R) and 5′-GCTGTTCTATGGAGGAACCCTCTACCCCCGGTA (MUT8-TSH-R; stop codons at nucleotide positions 2224 and 2239, shown underlined, in the reverse-complemented orientation). The arrows indicate the positions of the stops codons at which point the carboxyl terminus of the TSH receptor is truncated.

Figure 19B: TSH binding to CHO cells expressing the wild-type human TSH receptor and TSH receptor mutants MUT6-TSH-R, MUT7-TSH-R and MUT8-TSH-R and cells not expressing the TSH receptor (pSV2-neo). For details see the legend to Figure 1B.

Figure 19C: Cyclic AMP response to TSH stimulation in CHO cells expressing the wild-type human TSH receptor (WT-TSH-R) or the TSH receptor mutated in the cytoplasmic tail (see above). For details see the legend to Figure 1C.

Figure 20: Parallel inhibition by TSH and thyroid stimulating IgG of ^{125}I-TSH binding to

recombinant human TSH receptors stably expressed on the surface of Chinese hamster ovary (CHO) cells. Bovine TSH and DEAE-Sephadex-purified IgG (Methods) from the serum of a patients with Graves' disease containing potent thyroid stimulating activity was tested at the indicated concentrations for their ability to compete for ^{125}I-TSH binding, as described in Methods. As a control for the Graves' IgG, DEAE-purified IgG from a normal was also tested in the same assay. Each point represents the mean of closely agreeing values from duplicate dishes of cells.

Figure 21: Comparison of different TSH receptors in assays to detect anti-TSH receptor antibodies. Highly-purified IgG (DEAE-Sephadex ion exchange chromatography, see Methods) containing potent thyroid stimulating activity was tested at different dilutions with the indicated TSH receptor preparations. CHO-TSH-R - Chinese hamster ovary cells expressing recombinant human TSH receptors on their surface; FRTL5 rat thyroid cells; TRAK - commercially available solubilized porcine TSH receptor preparation. ^{125}I-TSH binding to CHO-TSH-R and FRTL5 cells in monolayer culture was performed as described in Methods. Each point represents the mean of closely agreeing values from duplicate dishes of cells, or duplicate solubilized receptor-containing tubes.

Figure 22: TSH binding inhibitory (TBI) activity in IgG prepared from the sera of patients with thyroid dysfunction and from normal individuals. A crude IgG fraction prepared by polyethylene glycol precipitation was added for 15 min prior to the addition of ^{125}I-TSH, as described in Methods. Data are normalized to an index, with 1.0 representing 100% of specific ^{125}I-TSH binding. A value of 0 would represent total

inhibition of ^{125}I-TSH binding by the IgG. ATD - antithyroid drugs (methimazole). Relapse - values obtained from sera drawn at the time of relapse of hyperthyroidism following the withdrawal of anti-thyroid drugs. Non-autoimmune thyroid disorders - 4 patients with toxic nodular goiter, 8 with single toxic adenomata, 3 with subacute thyroiditis and 3 with thyroid cancer. The numbers in parentheses indicate the number of patients in each group. Each point represents the mean of determinations in duplicate dishes of CHO-TSH-R cells. The horizontal dashed line represents the limit of inhibition of ^{125}I-TSH binding by any serum in the normal group (92% of maximum binding). The horizontal solid lines indicate the mean value for each group of patients.

Figure 23: Relationship between the TSH binding inhibition (TBI) index and thyroid stimulating immunoglobulin (TSI) bioactivity in the same IgG sample. IgG from 40 patients with untreated Graves' disease was prepared by polyethylene glycol precipitation (Methods). Bioactivity is expressed as the % of basal cAMP values in unstimulated human thyroid cell monolayer cultures. The shaded area represents a TBI index of <0.92 and a TSI value of >130% of the basal value.

Figure 24: Structures of chimeric TSH-LH/CG receptor extracellular regions and summary of the data obtained. The extracellular region of the human TSH receptor was divided into 5 arbitrary domains (A to E) as previously described (Example V). The open bars denote the human TSH receptor sequence and the black bars denote the rat LH/CG receptor sequence. Domain D in the LH/CG receptor is 50 amino acids smaller than its TSH receptor corresponding region (McFarland,

K.C., et al., Science 245:494-499 (1989); Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)) as shown by the thin horizontal line. Chimeric receptors are designated as TSH-LHR-12 through 16. Chimera TSH-LHR-11 was reported in the previous study (Example V) but is included here as a necessary control. Amino acid numbering is that used previously (Example V). The pluses represent an apprpoximation of the relative activities of the different chimeric receptors.

Figure 25A: Competition-inhibition by bTSH of [^{125}I]TSH binding to CHO cells expressing the wild-type human TSH receptor and chimeric TSH-LH/CG receptors. The binding of [^{125}I]TSH was measured in the presence of increasing concentrations of bovine TSH (bTSH), as described in Methods. Non-specific TSH binding was determined in the presence of 10^{-6} M unlabeled bTSH, and subtracted from each point to give the indicated value. These data represent one of two different experiments with pools of clones from two separate transfections, each transfection measured in duplicate dishes of cells.

Figure 25B: cAMP response to recombinant TSH (hTSH) stimulation in CHO cells expressing the wild-type human TSH receptor and chimeric TSH-LH/CG receptors. Incubation and cAMP assay was performed as described in Methods. Data are expressed as the percentage of values obtained in cells incubated under identical conditions in the absence of hTSH. The data represent one of two different experiments with pools of clones from two separate transfections; each point represents the mean of duplicate values determined in duplicate dishes of cells.

Figure 26A: Competition-inhibition of hCG of [^{125}I] hCG binding to CHO cells expressing the chimeric TSH-LH/CG receptors. Non-specific hCG binding was determined in the presence of 10^{-7} M unlabeled hCG and subtracted from each point to give the indicated value.

Figure 26B: cAMP response to hCG stimulation in CHO cells expressing chimeric TSH-LH/CG receptors.

Figure 27: Thyroid stimulating immunoglobulin (TSI) activity using selected TSH receptor/LH receptor chimeras. Basal activity is 100%. Stimulation is >100%. Purified IgG from 11 different Graves' disease sera were tested, as well as 4 Hashimoto's thyroiditis sera not containing TSI activity. The chimeras used have been described in Examples IX and VIII, and contain the indicated domains of the TSH receptor e.g. chimera TSH-LHR-6 contains TSH receptor domains A, B and C. WT TSH receptor represents the wild type (non-mutated) TSH receptor. ND indicates Not Done.

Figure 28: TSH Binding Inhibitory Immunoglobulin (TBII) activity using selected TSH receptor. LH receptor chimeras. TBII activity >15% is considered to be significant. See legend to Fig. 27 for details of chimeras and sera. Data for chimera TSH-LHR-10 are not shown because there was no activity, i.e. there was no inhibition of TSH binding by these IgG to this chimera.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following description, reference will be made to various methodologies known to those of skill in the art of molecular biology and immunology. Publications and other materials setting forth such

known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full.

Standard reference works setting forth the general principles of recombinant DNA technology include Watson, J.D. et al., Molecular Biology of the Gene, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell, J.E. et al., Molecular Cell Biology, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, B.M., Genes II, John Wiley & Sons, publishers, New York, N.Y. (1985); Old, R.W., et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, publisher, Berkeley, CA (1981); and Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, NY (1982).

By "cloning" is meant the use of in vitro recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ methods for generating DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

By "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

By "cDNA library" is meant a collection of recombinant DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library may be prepared by methods known to those of skill, and described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, supra. Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, thyroidal cell lines. A presently preferred vector for this purpose is the λ-ZAP II vector.

By "vector" is meant a DNA molecule, derived from a plasmid or bacteriophage, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites, and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible. Thus, by "DNA expression vector" is meant any autonomous element capable of replicating in a host independently of the host's chromosome, after additional sequences of DNA have been incorporated into the autonomous element's genome. Such DNA expression vectors include bacterial plasmids and phages.

By "substantially pure" is meant any antigen of the present invention, or any gene encoding any such antigen, which is essentially free of other antigens or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature. By "functional derivative" is meant the "fragments," "variants," "analogues," or "chemical derivatives" of

a molecule. A "fragment" of a molecule, such as any of the cDNA sequences of the present invention, is meant to refer to any nucleotide subset of the molecule. A "variant" of such molecule is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule is meant to refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Similarly, a "functional derivative" of a gene of the TSH receptor antigen of the present invention is meant to include "fragments," "variants," or

"analogues" of the gene, which may be "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity. Preferred functional derivatives include: recombinant active thyrotropin receptor wherein a 50 amino acid region comprising residues 317-366 has been deleted; a recombinant active thyrotropin receptor peptide comprised of the 8 amino acid region comprising residues 38-45; and a recombinant thyrotropin receptor peptide comprised of the mid-region domain C comprising amino acid residues 171-260.

Thus, as used herein, thyrotropin receptor (TSH) protein is also meant to include any functional derivative, fragment, variant, analogue, or chemical derivative thereof.

A DNA sequence encoding the thyrotropin receptor of the present invention, or its functional derivatives, may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Maniatis, T., et al., supra, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide.

An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the gene sequence coding for the protein may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and a thyrotropin receptor encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription

of the thyrotropin receptor gene sequence, or (3) interfere with the ability of the thyrotropin receptor gene sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express the protein, transcriptional and translational signals recognized by an appropriate host are necessary.

The present invention encompasses the expression of the thyrotropin receptor protein (or a functional derivative thereof) in either prokaryotic or eukaryotic cells, although eukaryotic (and, particularly, non-thyroidal eukaryotic) expression is preferred.

Preferred prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, etc. The most preferred prokaryotic host is E. coli. Other enterobacteria such as Salmonella typhimurium or Serratia marcescens, and various Pseudomonas species may also be utilized. Under such conditions, the protein may not be glycosylated. The procaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

To express the thyrotropin receptor protein (or a functional derivative thereof) in a prokaryotic cell (such as, for example, E. coli, B. subtilis, Pseudomonas, Streptomyces, etc.), it is necessary to operably link the TSH receptor encoding sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive promoters include the int

promoter of bacteriophage λ, the bla promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325, etc. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), the trp, recA, lacZ, lacI, and gal promoters of E. coli, the α-amylase (Ulmanen, I., et al., J. Bacteriol. 162:176-182 (1985)) and the σ-28-specific promoters of B. subtilis (Gilman, M.Z., et al., Gene 32:11-20 (1984)), the promoters of the bacteriophages of Bacillus (Gryczan, T.J., In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982)), and Streptomyces promoters (Ward, J.M., et al., Mol. Gen. Genet. 203:468-478 (1986)). Prokaryotic promoters are reviewed by Glick, B.R., (J. Ind. Microbiol. 1:277-282 (1987)); Cenatiempo, Y. (Biochimie 68:505-516 (1986)); and Gottesman, S. (Ann. Rev. Genet. 18:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold, L., et al. (Ann. Rev. Microbiol. 35:365-404 (1981)).

Most preferred hosts are eukaryotic hosts including yeast, insects, fungi, and mammalian cells either in vivo, or in tissue culture. Mammalian cells provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Mammalian cells which may be useful as hosts include cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/O-AG14 or the myeloma P3x63Sg8, and their derivatives. CHO-K1 cells are presently preferred mammalian host cells. COS cells also are

convenient eukaryotic hosts for thyrotropin receptor expression, as well as for study of the regulation of thyrotropin receptor expression.

For a mammalian cell host, many possible vector systems are available for the expression of TSH receptor. A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.

Yeast provides substantial advantages in that it can also carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

Further, by use of, for example, the yeast ubiquitin hydrolase system, in vivo synthesis of ubiquitin-TSH receptor fusion proteins may be accomplished. The fusion proteins so produced may be processed in vivo or purified and processed in vitro, allowing synthesis of the TSH receptor protein with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived methionine residues in direct yeast (or bacterial) expression may be avoided. Sabin et al., Bio/Technol. 7(7): 705-709 (1989); Miller et al., Bio/Technol. 7(7): 698-704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of TSH receptor or functional derivatives thereof in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express TSH receptor by methods known to those of skill. Thus, in one embodiment, sequences encoding human TSH receptor may be operably linked to the regulatory regions of the viral polyhedron protein (Jasny, Science 238: 1653 (1987)). Infected with the recombinant baculovirus, cultured insect cells, or the live insects themselves, can produce the TSH receptor protein in amounts as great as 20 to 50% of total protein production. When live insects are to be used, caterpillars are

presently preferred hosts for large scale TSH receptor production according to the invention.

As discussed above, expression of the thyrotropin receptor protein in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D., et al., J. Mol. Appl. Gen. 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., Cell 31:355-365 (1982)); the SV40 early promoter (Benoist, C., et al., Nature (London) 290:304-310 (1981)); the yeast gal4 gene promoter (Johnston, S.A., et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975 (1982); Silver, P.A., et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955 (1984)). Of these, presently the most preferred is the SV40 promoter.

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the TSH receptor protein (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as TSH receptor encoding DNA sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the TSH receptor encoding sequence).

The TSH receptor encoding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a

non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the TSH receptor protein may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome.

In one embodiment, a vector is employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotropic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., Mol. Cel. Biol. 3:280 (1983).

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector

include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Preferred prokaryotic vectors include plasmids such as those capable of replication in E. coli (such as, for example, pBR322, ColE1, pSC101, pACYC 184, πVX. Such plasmids are, for example, disclosed by Maniatis, T., et al. (In: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)). Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: The Molecular Biology of the Bacilli, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include pIJ101 (Kendall, K.J., et al., J. Bacteriol. 169:4177-4183 (1987)), and streptomyces bacteriophages such as φC31 (Chater, K.F., et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John, J.F., et al. (Rev. Infect. Dis. 8:693-704 (1986)), and Izaki, K. (Jpn. J. Bacteriol. 33:729-742 (1978)).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al., Miami Wntr. Symp. 19:265-274 (1982); Broach, J.R., In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, J.R., Cell 28:203-204 (1982); Bollon, D.P., et al., J. Clin. Hematol. Oncol.

10:39-48 (1980); Maniatis, T., In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Expression, Academic Press, NY, pp. 563-608 (1980)).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the vector or DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile (biolistic) bombardment (Johnston et al., Science 240(4858): 1538 (1988)), etc.

After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the TSH receptor protein, or in the production of a fragment of this protein. This can take place in the transformed cells as such, or following the induction of these cells to differentiate.

The expressed protein may be isolated and purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like. For example, the cells may be collected by centrifugation, or with suitable buffers, lysed, and the protein isolated by column chromatography, for example, on DEAE-cellulose, phosphocellulose, polyribocytidylic acid-agarose, hydroxyapatite or by electrophoresis or immunoprecipitation. Alternatively, the TSH receptor

or functional derivative thereof may be isolated by the use of anti-TSH receptor antibodies. Such antibodies may be obtained by well-known methods, some of which as mentioned hereinafter.

Yet another embodiment of the present invention comprises antibodies against the TSH receptor protein or a functional derivative thereof. The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab')_2$ fragments) which are capable of binding an antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods. For example, cells expressing the TSH receptor protein, or a functional derivative thereof, can be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding TSH receptor.

In a preferred method, antibodies according to the present invention are monoclonal antibodies. Such monoclonal antibodies can be prepared using hybridoma technology (Kohler et al., Nature 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal with TSH receptor antigen. The splenocytes of such animals are extracted and fused with a suitable myeloma cell line.

Any suitable myeloma cell line may be employed in accordance with the present invention. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands, J.R., et al. (Gastro-enterology 80:225-232 (1981). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the TSH receptor antigen.

Antibodies according to the present invention also may be polyclonal, or, preferably, region specific polyclonal antibodies. Region specific polyclonal antibodies and methods of using them are described in co-pending U.S. application Serial Number 06/731,470, filed 07 May 1985, the specification of which is incorporated herein by reference as though set forth in full.

Antibodies against TSH receptor a functional derivative thereof, according to the present invention are well suited for use in standard immunodiagnostic assays known in the art, including such immunometric or "sandwich" assays as the forward sandwich, reverse sandwich, and simultaneous sandwich assays. The antibodies may be used in any number of combinations as may be determined by those of skill without undue experimentation to effect immunoassays of acceptable specificity, sensitivity, and accuracy for the TSH receptor antigen or equivalents thereof.

Standard reference works setting forth general principles of immunology include Roitt, I., Essential Immunology, Sixth Ed., Blackwell Scientific Publications, Publisher, Oxford (1988); Kimball, J. W., Introduction to Immunology, Second Ed., Macmillan Publishing Co., Publisher, New York (1986); Roitt, I.,

et al., Immunology, Gower Medical Publishing Ltd., Publisher, London, (1985); Campbell, A., "Monoclonal Antibody Technology," in, Burdon, R., et al., eds., Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier, Publisher, Amsterdam (1984); Klein, J., Immunology: The Science of Self-Nonself Discrimination, John Wiley & Sons, Publisher, New York (1982); and Kennett, R., et al., eds., Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses, Plenum Press, Publisher, New York (1980).

By "detecting" it is intended to include determining the presence or absence of a substance or quantifying the amount of a substance. The term thus refers to the use of the materials, compositions, and methods of the present invention for qualitative and quantitative determinations.

The isolation of other hybridomas secreting monoclonal antibodies of the same specificity as those described herein can be accomplished by the technique of anti-idiotypic screening. Potocmjak, et al., Science 215:1637 (1982). Briefly, an anti-idiotypic antibody is an antibody which recognizes unique determinants present on the antibody produced by the clone of interest. The anti-idiotypic antibody is prepared by immunizing an animal of the same strain used as the source of the monoclonal antibody with the monoclonal antibody of interest. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing antibody to these idiotypic determinants (anti-idiotypic antibody).

By using the anti-idiotypic antibody of the second animal, which is specific for the monoclonal

antibodies produced by a single clone, it is then possible to identify other clones used for immunization. Idiotypic identity between the product of two clones demonstrates that the two clones are identical with respect to their recognition of the same epitopic determinants. The anti-idiotypic antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti anti-idiotypic antibody which will be epitopically identical to the original MAb. Thus, by using antibodies to the epitopic determinants of a monoclonal antibody, it is possible to identify other clones expressing antibodies of identical epitopic specificity. In antibodies, idiotypic determinants are present in the hypervariable region which binds to a given epitope.

Accordingly, monoclonal antibodies generated against the TSH receptor antigen or functional derivative thereof may be used to induce anti-idiotypic Abs in suitable animals, such as BALB/c mice. Spleen cells from these animals are used to produce anti-idiotypic hybridoma cell lines. Monoclonal anti-idiotypic Abs coupled to KLH are used as "immunogen" to immunize BALB/c mice. Sera from these mice will contain anti anti-idiotypic Abs that have the binding properties of the original Ab specific for the shared epitope. The anti-idiotypic MAbs thus have idiotopes structurally similar to the epitope being evaluated.

For replication, the hybrid cells may be cultivated both in vitro and in vivo. High in vivo production makes this the presently preferred method of culture. Briefly, cells from the individual hybrid strains are injected intraperitoneally into pristane-

primed BALB/c mice to produce ascites fluid containing high concentrations of the desired monoclonal antibodies. Monoclonal antibodies of isotype IgM or IgG may be purified from cultured supernatants using column chromatography methods well known to those of skill in the art.

Antibodies according to the present invention are particularly suited for use in immunoassays wherein they may be utilized in liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways.

There are many different labels and methods of labeling known in the art. Examples of the types of labels which can be used in the present invention include, but are not limited to, enzymes, radioisotopes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds and metal chelates. Those of ordinary skill in the art will know of other suitable labels for binding to antibodies, or will be able to ascertain the same by the use of routine experimentation. Furthermore, the binding of these labels to antibodies can be accomplished using standard techniques commonly known to those of ordinary skill in the art.

One of the ways in which antibodies according to the present invention can be detectably labeled is by linking the antibody to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected as, for example, by spectrophotometric or fluorometric means. Examples of enzymes which can be used to detectably label antibodies include malate dehydrogenase,

staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotin-avidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase.

The presence of detectably labeled antibodies also can be detected by labeling the antibodies with a radioactive isotope which then can be determined by such means as the use of a gamma counter or a scintillation counter. Isotopes which are particularly useful for the purpose of the present invention are ^{3}H, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe and ^{75}Se.

It is also possible to detect the binding of detectably labeled antibodies by labeling the antibodies with a fluorescent compound. When a fluorescently labeled antibody is exposed to light of the proper wave length, its presence then can be detected due to the fluorescence of the dye. Among the most commonly used fluorescent labeling compounds are fluoroscein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibodies of the invention also can be detectably labeled using fluorescent emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

Antibodies also can be detectably labeled by coupling them to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of the chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salts, oxalate ester , and dioxetane.

Likewise, a bioluminescent compound may be used to label the antibodies according to the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent antibody is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling include luciferin, luciferase and aequorin.

The antibodies and substantially purified antigen of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of said container means comprising the separate elements of the assay to be used.

The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunometric, or sandwich, immunoassays.

By the term "immunometric assay" or "sandwich immunoassay," it is meant to include simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

Forward sandwich assays are described, for example, in United States Patents 3,867,517; 4,012,294 and 4,376,110. Reverse sandwich assays have been described, for example, in United States Patents 4,098,876 and 4,376,110.

In the preferred mode for preforming the assays it is important that certain "blockers" be present in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, protease, or human antibodies to mouse immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore adds substantially to the specificity of the assays described in the present invention.

It has been found that a number of nonrelevant (i.e. nonspecific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g. IgG_1, IgG_{2a}, IgM, etc.) can be used as "blockers." The concentration of the "blockers" (normally 1-100 microgs/microl) is important, in order to maintain the

proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in human serum. In addition, the buffer system containing the "blockers" needs to be optimized. Preferred buffers are those based on weak organic acids, such as imidazole, HEPPS, MOPS, TES, ADA, ACES, HEPES, PIPES, TRIS, and the like, at physiological pH ranges. Somewhat less preferred buffers are inorganic buffers such as phosphate, borate or carbonate. Finally, known protease inhibitors should be added (normally at 0.01-10 microgs/ml) to the buffer which contains the "blockers."

There are many solid phase immunoadsorbents which have been employed and which can be used in the present invention. Well known immunoadsorbents include glass, polystyrene, polypropylene, dextran, nylon and other materials, in the form of tubes, beads, and microtiter plates formed from or coated with such materials, and the like. The immobilized antibodies can be either covalently or physically bound to the solid phase immunoadsorbent, by techniques such as covalent bonding via an amide or ester linkage, or by adsorption. Those skilled in the art will know many other suitable solid phase immunoadsorbents and methods for immobilizing antibodies thereon, or will be able to ascertain such, using no more than routine experimentation.

For in vivo, in vitro or in situ diagnosis, labels such as radionuclides may be bound to antibodies according to the present invention either directly or by using an intermediary functional group. An intermediary group which is often used to bind radioisotopes which exist as metallic cations to antibodies is diethylenetriaminepentaacetic acid (DTPA).

Typical examples of metallic cations which are bound in this manner are: ^{99m}Tc, ^{123}I, ^{111}IN, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga and ^{68}Ga. The antibodies of the invention can also be labeled with non-radioactive isotopes for purposes of diagnosis. Elements which are particularly useful in this manner are ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr and ^{56}Fe.

The DNA sequences which encode TSH receptor, or a fragment thereof, may be used as DNA probes to isolate the corresponding antigen in humans according to well-known methods. The human antigen genes may then be cloned and expressed in a host to give the human antigen. This human antigen may then be used in diagnostic assays for the corresponding auto-antibody.

The antigen of the invention may be isolated in substantially pure form employing antibodies according to the present invention. Thus, an embodiment of the present invention provides for substantially pure antigen TSH receptor or functional derivative thereof, said antigen characterized in that it is recognized by and binds to antibodies according to the present invention. In another embodiment, the present invention provides a method of isolating or purifying the TSH receptor antigen, by forming a complex of said antigen with one or more antibodies directed against TSH receptor or functional derivative thereof.

The substantially pure antigen TSH receptor or functional derivative of the present invention may in turn be used to detect or measure antibody to TSH receptor in a sample, such as serum or urine. Thus, one embodiment of the present invention comprises a method of detecting the presence or amount of antibody to TSH receptor antigen in a sample, comprising contacting said sample containing said antibody to TSH

receptor antigen with detectably labeled TSH receptor, and detecting said label. It will be appreciated that immunoreactive fractions and immunoreactive analogues of TSH receptor also may be used. By the term "immunoreactive fraction" is intended any portion of the TSH receptor antigen which demonstrates an equivalent immune response to an antibody directed against TSH receptor. By the term "immunoreactive analogue" is intended a protein which differs from the TSH receptor protein by one or more amino acids, but which demonstrates an equivalent immunoresponse to an antibody of the invention.

The pharmaceutical compositions of the invention may be administered to any animal which may experience the beneficial effects of the compounds of the invention. Foremost among such animals are humans, although the invention is not intended to be so limited.

The pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

In addition to the pharmacologically active compounds, the new pharmaceutical preparations may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations

which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.01 to 99 percent, together with the excipient.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Suitable excipients are, in particular, fillers such as saccharides, for example lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate.

Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetyl-cellulose phthalate or hydroxypropymethyl-cellulose phthalate, are used. Dye stuffs or pigments may be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, or liquid paraffin. In addition, stabilizers may be added.

Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of one or more of the active compounds with a suppository base. Suitable

suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The substantially pure antigen TSH receptor of the present invention may be used in pharmacologically effective amounts in pharmaceutical preparations to treat thyrotoxicosis. In Graves' disease, the most common form of thyrotoxicosis, anti-TSH receptor auto-antibodies bind to TSH receptor on the thyroid cell surface. Such binding causes unregulated stimulation of the thyroid cell which then produces excessive amounts of thyroid hormone. Tolerance to anti-TSH receptor auto-antibodies may be induced in patients by the injection of pharmaceutically effective amounts of recombinant TSH receptor, or a functional or chemical derivative thereof. As more recombinant TSH receptor

is introduced, a higher percentage of the auto-antibodies present combine with the recombinant TSH receptor as opposed to the naturally occurring TSH receptor on the thyroid cell surface. As less auto-antibodies combine with the cell bound TSH receptor, the thyroid cells are less stimulated and thyroid hormone production becomes subject to the regulation of thyrotropin.

Autoimmune diseases are thought to occur when thymus-derived lymphocytes (T cells) become persistently activated by self antigens (Janeway, C., Nature 341: 482 (1989)). In the case of autoimmune thyrotoxicosis, this self antigen is the epitope of the TSH receptor which is recognized by a receptor on the autoimmune T cells. The present invention allows the determination of this epitope of the TSH receptor using standard techniques commonly known to those of ordinary skill in the art. Further, the present invention makes possible the characterization of the autoimmune T-cell receptor (TCR) specific to the TSH receptor using methods described in, for example, Burns, F., et al., J. Exp. Med. 169: 27 (1989). If the autoimmune T cells can be eliminated or prevented from reacting with the TSH receptor, the effects of thyrotoxicosis may be greatly alleviated. T cells that will accomplish this objective may be generated which are specific for the autoimmune TCR for TSH receptor using methods described in, for example, Acha-Orbea, H., et al., Ann. Rev. Immunol. 7: 371 (1989).

Thus in one embodiment of the invention, a T cell is provided which is specific for the autoimmune TCR for TSH receptor. Another embodiment of the invention provides for a pharmaceutical preparation comprising

a T cell which is specific for the autoimmune TCR for TSH receptor. In yet another embodiment of the current invention, a method of treating autoimmune thyrotoxicosis is provided comprising the use of a pharmaceutical preparation comprising a T cell which is specific for the autoimmune TCR for TSH receptor.

Upon characterization of the autoimmune TCR for TSH receptor, peptides may be generated to interfere with the autoimmune TCR using methods described in, for example, Vandenbark, A., et al., Nature 341: 541 (1989) and Janeway, C., Nature 341: 482 (1989). Such interference would reduce the interaction between the autoimmune T cells and the TSH receptor, greatly reducing the effects of autoimmune thyrotoxicosis.

Thus in another embodiment of the invention, a peptide is provided which is specific for the autoimmune TCR for TSH receptor. Another embodiment of the invention provides for a pharmaceutical preparation comprising a peptide which is specific for the autoimmune TCR for TSH receptor. In yet another embodiment of the current invention, a method of treating autoimmune thyrotoxicosis is provided comprising the use of a pharmaceutical preparation comprising a peptide which is specific for the autoimmune TCR for TSH receptor.

A class of T cells, known as suppressor T cells, mediate the immunity suppressor system. These cells provide a mechanism for turning off immune responses that otherwise might overwhelm the host.

When an animal is exposed to an excess of antigen to which it is genetically nonresponsive, several events take place. Benacerraf, B., In: The Biology of Immunologic Disease, HP Publishing Co., Inc., NY, pp. 49-62 (1983). Antigen first induces a population of

suppressor T cells. These lymphocytes elaborate a soluble factor that combines with small quantities of antigen and induces a second suppressor T-cell clone. It is these effector cells that actually suppress the antibody-producing B cells. It is likely that this system serves the homeostatic role of turning off antibody response that threatens to overwhelm the host.

To treat thyrotoxicosis, animals are exposed to excess amounts of thyrotropin receptor and the resulting suppressor T cells specific for anti-thyrotropin receptor auto-antibodies are harvested using methods as described in, for example, Green, D., et al., Ann. Rev. Immunol. 1: 439 (1983) or Benacerraf, B., In: The Biology of Immunologic Disease, HP Publishing Co., Inc., NY, pp. 49-62 (1983). Patients with thyrotoxicosis are treated with the suppressor T cells so as to suppress the formation of anti-thyrotropin receptor auto-antibodies. The suppression of the auto-antibody production would greatly lessen or eliminate the effects of thyrotoxicosis.

Thus in one embodiment of the invention, a suppressor T cell is provided which is specific for anti-thyrotropin receptor auto-antibodies. Another embodiment of the invention provides for a pharmaceutical preparation comprising a suppressor T cell which is specific for anti-thyrotropin receptor auto-antibodies. In yet another embodiment of the current invention, a method of treating autoimmune thyrotoxicosis is provided comprising the use of a pharmaceutical preparation comprising a suppressor T cell which is specific for anti-thyrotropin receptor auto-antibodies.

The manner and method of carrying out the present invention may be more fully understood by those of skill by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

EXAMPLE I

Isolation and characterization of a cDNA clone for the human TSH receptor: A human thyroid (Graves' disease) cDNA library was constructed in the Eco RI site of lambda-ZAP II (Stratagene, San Diego, CA) using the Amersham (Arlington Heights, IL) cDNA Synthesis kit. mRNA for cDNA synthesis was prepared (9) from a thyroid gland removed, for appropriate medical indications, from a patient with Graves' disease. The library was screened with two synthetic oligonucleotides (GTGTTCAGGTCCGAGCTGTCCGTGTACACCCTG-ACCGTGATCACCCTGGAGAGGTGGTA and CACGCCTGCAGGATCATGGTG-GGCGGCTGGGTGTGCTGCTTCCTGCTGGCC) end-labeled with gamma ^{32} P-ATP and polynucleotide kinase (10). The low stringency screening method of Wood was utilized (11), with the exception that final washes were at 42°C in 0.2 x SSC, 0.1% SDS. Nucleotide sequence of selected clones was determined by the dideoxynucleotide chain termination method (12) using modified bacteriophage T7 DNA polymerase (Sequenase, U.S.B. Cleveland, OH). Double-stranded Bluescript SK(-) plasmids, generated from the lambda-ZAP vector using R408 helper phage, were used as templates.

EXAMPLE II

Functional expression of the TSH receptor: The cDNA insert in clone 4 (approximately 4 kb in length) was excised from Bluescript with Eco RI and ligated into the Eco RI site of the eukaryotic expression plasmid pSV2-NEO-ECE. This plasmid was constructed by the combination of pECE (13) with pSV2-NEO (14). In brief, the Eco RI site in pSV2-NEO was eliminated by religation after blunting of the ends with the Klenow fragment of DNA polymerase I. pECE was cut with Pvu II, and Bam HI linkers were ligated onto the blunted ends. Bam HI digestion of the linkers, as well as of an internal Bam HI site in pECE, released the functional region of this vector which was then ligated into the Bam HI site of pSV2-NEO The plasmid pSV2-NEO-ECE-hTSHR, containing the putative TSH receptor gene in the correct orientation, was stably transfected into CHO-K1 cells (15). Following selection with G418 (400 ug/ml), surviving clones were pooled and subcloned into 2 cm^2 dishes. Cells were exposed (1 h at 37°C) to the indicated hormones in culture medium (F12, containing 10% fetal calf serum and standard antibiotics) to which was also added 1 mM 3-isobutyl-1-methylxanthine. After aspiration of the medium, cellular cAMP was extracted with ethanol and measured by radioimmunoassay, as previously described (16).

DISCUSSION

Initial attempts to isolate a cDNA clone for the rat TSH receptor, using low-stringency PCR screening of a rat thyroid cDNA library and synthetic

oligonucleotides based on the reported nucleotide sequence of the extracellular domain of the rat LH\CG receptor (6), were unsuccessful. Consequently two oligonucleotides, based on the reported amino acid sequence of the 3rd and 4th transmembrane domains of a putative human TSH receptor and related receptors (8), were used to screen a human thyroid cell cDNA library. Twenty eight clones were obtained from 5 x 10^5 screened. The nucleotide sequence, and derived amino acid sequence, of selected cDNA clones confirmed the amino acid sequence of the region on which the oligonucleotides were based. Three of 4 clones were full-length in that they contained polyadenylated tails and the putative ATG initiation codon downstream of in-frame stop codons. The length on gel electrophoresis of these 3 clones was approximately 4 kb, similar to that of the LH\CG receptor (6, 7).

The translated region of one of these cDNA clones (clone 4) was determined in full in both directions (Fig. 1). This clone contains an open reading frame of 764 amino acids, with a calculated molecular weight of 86,816 Daltons. A hydrophobicity plot (17) of this derived amino acid sequence reveals a putative signal peptide and seven transmembrane domains (Fig. 2). The extracellular domain spans approximately 418 amino acids, and contains five potential glycosylation sites. There is only 33% amino acid homology with the corresponding extracellular domain of the pig LH\CG receptor (7) (Fig. 3). The transmembrane region of the human TSH receptor is approximately 264 amino acids in length (residues 419-682). There is greater homology (71%) between the transmembrane regions of the human TSH receptor and the pig LH\CG receptor, with 100% homology in the third transmembrane domain,

on whose amino acid sequence one of the oligonucleotide probes was based. The short intracytoplasmic domain of approximately 82 amino acids (residues 683-764) bears little homology to the corresponding region of the LH\CG receptor.

Functional analysis was performed in order to confirm the identity of cDNA clone 4 as the putative TSH receptor. This cDNA was ligated in the correct orientation into the eukaryotic expression vector pSV2-NEO-ECE. The resulting plasmid (pSV2-NEO-ECE-hTSHR) was transfected into Chinese hamster ovary (CHO) cells. TSH (10 mU/ml, equivalent to 10^{-8} M) stimulation of pooled colonies of stable transfectants increased intracellular cAMP levels 5-fold (Fig. 4). A cAMP response in transfected cells was initially evident at 0.33 mU/ml (3.3×10^{-10} M) TSH. No effect of TSH on cAMP levels was observed in control, untransfected CHO cells, or in transfected cells stimulated with 10^{-7} M hCG, (1-24)ACTH or insulin.

EXAMPLE III

AN 8 AMINO ACID "INSERTION" IN THE HUMAN TSH RECEPTOR IS CRITICAL FOR HIGH AFFINITY TSH BINDING

Comparison of the primary amino acid sequences of the TSH and 1H/C; receptors revealed two unique "insertions" of 8 and 50 amino acids in the extracellular domain of the TSH receptor. The functional significance of each was determined by site-directed mutagenesis. Deletion of the 50 amino acid tract (residues 317-366) resulted in no effect on TSH binding or on TSH or thyroid stimulating imuunoglobulin (TSI) biological activity. In contrast, either deletion or substitution of the 8

amino acid region (residues 38-45) abolished these activities. This 8 amino acid tract near the amino terminus of the TSH receptor is a uniquely important site of interaction for both TSH and TSI.

Thyrotropin (TSH) regulates thyroid cell function by interacting with the TSH receptor on the plasma membrane (Dumont et al., Adv. Cyclic Nucleotide Res. 14:479 (1981)). The sites on the TSH receptor which interact with TSH and TSI are unknown. The molecular cloning and determination of the primary amino acid sequences of the TSH (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989); Libert et al., Biochem. Biophys. Res. Comm. 165:1250 (1989); Misrahi et al., Biochem. Biophys. Res. Comm. 166:394 (1990); Parmentier et al., Science 246:1620 (1989)) and LH/CG (McFarland et al., Science 245:494 (1989); Loosfelt et al., Science 245:525 (1989)) receptors has revealed two unique "insertions" of 8 and 50 amino acids in the extracellular domain of the 764 amino acid TSH receptor relative to that of the LH/CG receptor (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989)). We have therefore focused on these two regions using site-directed mutagenesis to determine their functional significance.

METHODS

The full-length human TSH receptor cDNA in Bluescript (Stratagene, San Diego, CA) was subjected to oligonucleotide-directed mutagenesis (Muta-gene kit, Bio-Rad, Richmond, CA) (Kunkel, T.A., Proc. Natl. Acad. Sci. USA 74:5463 (1977)). Nucleotides coding for amino acids 38-45 and 317-366 were deleted, separately and in combination, using oligonucleotides

with bases complementary to the nucleotides flanking these regions (CGGTAAGCTGGGGATGCGGAAGTCCTCCTCCTGATG and GGGTTTTTGAGCTCCTGGCCATTCACAGATTTTCTCTGGC, respectively). The amino acids at 38-45 (RVTCKDIQ) were substituted with the sequence SASSSSAS using the oligonucleotide AAGCTGGGGATGCGTGAAGCAGAACTGCTGGACG CACTGAAGTCCTCCTCCT, containing 14 bases complementary to the nucleotides flanking either side of the substituted sequence. The nucleotide sequences of the mutagenized and adjacent regions were determined (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977)) and confirmed to be correct. The mutagenized TSH receptor cDNAs were excised with Eco RI and subcloned into the expression vector pSV2-NE0-ECE (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989)). These mutants, as well as the wild-type receptor in the same vector and a vector without a cDNA insert as controls, were transfected by the calcium-phosphate method (Chen et al., Mol. Cell. Biol. 7:2745 (1987)) into CH0 cells. Surviving colonies were selected by G418 (400 μg/mL) and pooled for study of their ability to bind to TSH and to respond to TSH with respect to an increase in intracellular cAMP levels.

TSH binding studies were performed as previously described (Chazenbalk et al., Endocrinology (in press) (1990)) with the exception that TSH was iodinated with ^{125}I to approximately 80 uCi/ug protein using the Bolton-Hunter reagent. Non-specific ^{125}I-TSH binding was determined in the presence of $10^{-8}M$ TSH and this value was subtracted from total binding to yield specific TSH binding. Measurements of the intracellular cAMP response to hormone stimulation (1 hr at

37°C) was as previously described (Hirayu et al., Molec. Cell. Endocrinol. 42:21 (1985)).

RESULTS

The cDNA coding for the two unique segments in the extracellular domain of the human TSH receptor, residues 38-45 and residues 317-366 (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989)), were deleted by site-directed mutagenesis, singly or together (Fig. 5). These segments are near the amino and carboxyl termini of the 418 amino acid TSH receptor extracellular domain (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989)), respectively (Fig. 5). Chinese hamster ovary cells were stably transfected with these mutated TSH receptor cDNAs, which were then tested for their ability to bind TSH and to respond to TSH stimulation in terms of an increase in intracellular cAMP content.

Surprisingly, even though the 50 amino acid deletion (hTSHR-D1) represents 12% of the 418 amino acid extracellular domain of the TSH receptor, high affinity TSH binding to the human TSH receptor was not reduced (Table I). Consistent with this high affinity TSH binding, TSH stimulated cAMP generation in cells expressing hTSHA-D1 with an EC50 similar to that of cells with the wild-type receptor. In contrast to the 50 amino acid deletion in hTSHR-D1, the deletion of 8 amino acids near the amino terminus (hTSHR-M2) completely abolished high affinity TSH binding. Again consistent with absent high affinity TSH binding, TSH stimulation did not increase intracellular cAMP concentrations. Not unexpectedly, CHO cells transfected with a TSH receptor cDNA in which both the

50 and the 8 amino acid segments were deleted (hTSHR-D3) had neither high affinity TSH binding nor a cAMP response to TSH stimulation.

Because the 8 amino acid deletion in hTSHR-M2 might have resulted from a conformational change resulting from abnormal protein folding rather than the loss of a critical hormone binding site in the receptor, we constructed a mutant receptor (hTSHR-S1) in which the 8 amino acids were not deleted, but were instead substituted with 8 residues (Fig. 5) that would not be expected to alter the generally hydrophilic nature of this region and its likely presence on the surface of the globular protein. There is evidence that normal folding may be unaffected by substantial alterations of hydrophilic regions at the surface of globular proteins (Bowie et al., Science 247:1306 (1990)), as is evident from the 50 amino acid deletion in hTSHR-D1. As with the deletion mutation in this region (hTSHR-D2), hTSHR-S1 expressed neither high affinity TSH binding nor a cAMP response to TSH stimulation (Table I).

Receptor	High Affinity TSH Binding Kd(nM)	Kd-M/ Kd-Wt	TSH Stimulation of cAMP C50(nM)	EC50-M/ EC50-Wt
hTSHR-WT	0.30	1	4.0	1
hTSHR-D1	0.20	0.7	7.0	1.4
hTSHR-D2	n.d.	n.d.	n.d.	n.d.
hTSHR-D3	n.d.	n.d.	n.d.	n.d.
hTSHR-S1	n.d.	n.d.	n.d.	n.d.
pSV2-NE0-ECE	n.d.	n.d.	n.d.	n.d.

Table I: Hormone binding and function in CH0 cells stably expressing the TSH receptor mutants depicted in Fig. 1. WT - wild type. pSV2-NE0-ECE is the vector without cDNA insert.
* - not detectable.

Each value represents the mean of data obtained with pools of clones from two separate transfections, each transfection measured in duplicate.

Sera from patients with Graves' disease containing thyroid stimulating immunoglobulin (TSI) stimulated the mutant TSH receptor clones with the same profile as did TSH (Fig. 6). Thus, only the wild-type TSH receptor and the TSH receptor with the 50 amino acid deletion (hTSHR-D1) exhibited a cAMP response to a pool of 4 sera with potent TSI activity as measured in the conventional TSI bioassay (Hinds et al., J. Clin. Endocrinol. Metab. 52:1204 (1981)). Deletion or substitution of the 8 amino acid tract (residues 317-366) in the TSH receptor abolished the cAMP response to TSI stimulation. These data indicate that the 50 amino acid tract does not contain an epitope important for TSI action. In contrast, as for TSH action, the 8 amino acid segment near the amino terminus of the receptor contains an important site of interaction for TSI.

Finally, because 8 and 50 amino acid segments in the TSH receptor are absent from the LH/CG receptor (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989); Loosfelt et al., Science 245:525 (1989)) it was conceivable that their deletion from the TSH receptor would alter the hormone specificity of the TSH receptor and allow it to respond to hCG stimulation in terms of a cAMP response. This was not the case. As with the wild-type TSH receptor, the foregoing mutant TSH receptors were unresponsive to 10^{-7}M hCG (data not shown).

The pituitary/placental glycoprotein hormone receptors represent a distinct subgroup in the G protein-coupled receptor family with seven transmembrane spanning regions. The majority of the members of this receptor family, such as the α-adrenergic, β-adrenergic, muscarinic acetyl choline and dopamine receptors (which we term group A), interact with small ligands and have insignificant extracellular regions. In contrast, the receptors for the very large glycoprotein hormones TSH, LH, CC and FSH (group B) have large extracellular domains (348-418 amino acids) (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989); Libert et al., Biochem. Biophys. Res. Comm. 165:1250 (1989); Misrahi et al., Biochem. Biophys. Res. Comm. 166:394 (1990); Loosfelt et al., Science 245:525 (1989); Sprengel et al., Mol. Endocrinol. 4:525 (1990)), the largest of which is the TSH receptor, primarily because of the presence of the 50 amino acid "insertion". It is remarkable that this 50 amino acid tract unique to the TSH receptor is not important in TSH binding or on signal transduction (cAMP generation). Therefore, it was possible that this region served a negative function, by preventing

the binding of other ligands to the TSH receptor. For this reason the inventors determined whether its deletion would allow the receptor to respond to hCG. However, this was not the case. In contrast to the 50 amino acid tract, the unique 8 amino acid region near the amino terminus of the TSH receptor extracellular domain is functionally significant. The present data therefore provide strong evidence for the localization of a site for TSH binding to the TSH receptor. Interestingly, the amino acid sequence of the rat FSH receptor, reported after the completion of these studies (Sprengel et al., Mol. Endocrinol. 4:525 (1990)), reveals a unique 10 amino acid segment in the same general region (residues 21-30) as the 8 amino acid segment in the hTSH receptor but, like the receptor, does not contain the unique 50 amino acid segment of the TSH receptor (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184 (1989); Sprengel et al., Mol. Endocrinol. 4:525 (1990)). The significance of this FSH receptor segment remains to be determined.

EXAMPLE IV

ASPARAGINE-LINKED OLIGOSACCHARIDES AT SPECIFIC SITES OF THE THYROTROPIN RECEPTOR ARE ESSENTIAL FOR RECEPTOR FUNCTION

The role of glycosylation on ligand binding and signal transduction in the human thyrotropin (TSH) receptor was studied. The amino acid sequence deduced from the recently isolated human TSH receptor cDNAs (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); Libert et al., Biochem. Biophys. Res. Comm. 165:1250-1255 (1989); Misrahi et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990)) indicates the presence of six potential sites of N-

linked glycosylation, all in the extracellular domain of the receptor. To investigate the contribution of each N-linked oligosaccharide chain in the functional activity of the receptor we used oligonucleotide-directed mutagenesis to replace, separately or together, the Asn codons in each of the 6 potential glycosylation sites with Gln.

This mutagenesis approach has permitted us to examine the contribution of each potential N-linked glycosylation site in hormone binding and signal transduction, without the disadvantages associated with glycosylation inhibitors or deglycosylating enzymes, such as damage to the protein backbone or incomplete removal of the carbohydrate noieties. The data obtained suggest that N-linked glycosylation at ssparagine (Asn) 77 and Asn 113 is required for the functional activity of the human TSH receptor.

METHODS

Site-directed mutagenesis and expression of the human TSH receptor. The full-length human TSH receptor cDNA in Bluescript (Stratagene, San Diego, CA) was subjected to oligonucleotide-directed mutagenesis (Mutagene phagemid in vitro mutagenesis kit, Bio-Rad, Richmond, CA) based on the methods described by Kunkel (Kunkel, T.A. Proc. Natl. Acad. Sci. USA 82:488-492 (1985)). Six oligonucleotides of 27 bases each were designed to replace the codons for Asn at position 77, 99, 113, 177, 198, 302 with glutamine codons. Oligonucleotides were synthesized by the Molecular Biology Resource Center, University of California, San Francisco. The mutant cDNA for the construct TSHAG1nx6 (with all six potential glycosylated sites mutated)

was obtained by using all six oligonucleotides in the same annealing reaction. The nucleotide sequence of the mutagenized and adjacent regions were determined (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977); Tabor et al., Proc. Nstl. Aced. Sci. USA 84:4767-4771 (1987)) and confirmed to be correct. The mutagenized TSH receptor cDNAs were excised with Eco RI and subcloned into the expression vector pSV2neo-ECE (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)). The mutants, as well as the wild-type receptor in the same vector and a vector without a cDNA insert as controls, were transfected by the calcium-phosphate method (Chen et al., Mol. Cell. Biol. 7:2745-2752 (1987)) into Chinese hamster ovary (CHO) cells. Surviving colonies (>100/dish) were selected by G418 (400 isg/ml) and pooled for study of their ability to bind to TSH and to respond to TSH with respect to an increase in intracellular cAMP levels.

Radiolabeled TSH binding. Cells grown to confluence (24-well Costar plates) in Ham's F-12 medium supplemented with 10% fetal calf serum and antibiotics were incubated for 2 hours at 37°C in 0.25 ml modified Hank's buffer without NaCl with isotonicity maintained with 280 mM sucrose, supplemented with 0.25% bovine serum albumin, ^{125}I-TSH (~1 x 10^{-4} cpm) and the indicated concentrations of unlabeled bovine TSH (Sigma Chemical Co., St. Louis), as previously described (Chazenbalk et al., Endocrinology (in press) (1990)). The bTSH (5 μg, 30 U/mg protein) was radiolabeled to a specific activity of approximately 80 μCi/μg protein with the Bolton-Hunter reagent (4400 Ci/mmol; New England Nuclear, Boston, MA) according to the protocol of the manufacturer, followed by Sephadex

G 100 chromatography. At the end of the incubation period, the cells were rapidly rinsed three times with the same buffer (ice-cold) without TSH, solubilized with 1 ml IN NaOH and radioactivity was measured in a gamma counter. Non-specific ^{125}I-TSH binding was determined in the presence of 10^{-6}M TSH and this value (<10% of total binding) was subtracted from total binding to yield specific TSH binding.

Cellular cAMP measurements. Transfected cells in 2 cm^2 dishes (Costar, Cambridge, MA) were incubated with fresh Ham's F-12 medium containing 1.5% BSA, 1 mM isobutyl methylxanthine (IBMX), and the indicated concentrations of TS]]. After 1 hour at 37°C, cellular cAMP was extracted with 95% ethanol and measured by radioimmunoassay as previously described (Hirayu et al., Molec. Cell. Endocrinol. 42:21-27 (1985)).

RESULTS

Seven mutant human TSH receptor cDNAs were synthesized: six cDNAs each lacking one of the potential sites of glycosylation (constructs TSHR-Gln77, TSHR-Gln99, TSHR-Gln113, TSHR-Gln177, TSHR-Gln198, TSHR-Gln302), and one cDNA lacking all six sites (TSHR-Glnx6) (Fig. 7B). These cDNAs, inserted into the eukaryotic expression vector pSV2neo-ECE (Nagayama et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)), were used to stably transfect CHO cells. Northern blot analysis of transfected cells probed with the human TSH receptor cDNA revealed bands of the appropriate size (4 kb) (data not shown).

We first examined the mutant TSH receptors for their TSH binding affinity. CHO cells transfected with the constructs TSHR-Gln99, TSHR-GlnI77, TSHR-Gln198

and TSHR-Gln302, demonstrated TSH binding with affinities (Kd 1.2 - 4 x 10^{-10}M) similar to cells transfected with the wild-type TSH receptor (Kd 3.3 x 10^{-10}M) (Fig. 8A,B, Table 2). However, TSHR-Gln113 showed a decrease in the affinity for TSH binding when compared to the wild-type TSH receptor (Kd 2.9 x 10^{-8}M) (Fig. 8C and Table 2).

TSH Receptor	TSH Binding Kd(M)
WT TSHR	3.3 ± 0.5 x 10^{-10}
TSHR-Gln 77	UND
TSHR-Gln 99	4.0 ± 0.7 x 10^{-10}
TSHR-Gln 113	2.6 ± 1.2 x 10^{-8}
TSHR-Gln 177	3.2 ± 0.6 x 10^{-10}
TSHR-Gln 198	3.9 ± 0.9 x 10^{-10}
TSHR-Gln 302	1.2 ± 0.4 x 10^{-10}
TSHR-Glnx6	UND

Table 2. TSH binding in CHO cells expressing the wild-type and the mutant TSH receptors. Each value represents the mean ± S.E. of data obtained with pool of clones from three separate experiments, each one measured in duplicate. WT TSHR - wild-type TSH receptor; UND - undetectable.

The greatest effects on TSH binding were observed with the mutation at Asn 77 (construct TSHR-Gln77) or when all the six potential glycosylation sites were mutated (TSHR-Glnx6). These mutants were associated with the complete loss of high affinity binding for the ligand (Fig. 8D). As expected, no high affinity TSH binding was detected in the CHO cells transfected with pSV2-neo only (Fig. 8C).

The TSH receptor mutants were further characterized for their biological activity in transducing

the hormonal signal as measured by an increase in intracellular cAMP levels. CHO cells expressing TSHR-Gln99, TSHR-Gln177, TSHRGln198 and TSHR-Gln302 all responded to TSH stimulation. The dose responses were similar among these mutant receptors, with an initial response evident at approximately 10^{-9}M TSH and a 3 - 5-fold increase in intracellular cAMP levels at 10^{-7}M TSH (Fig. 9A). In contrast, a complete loss of signal transduction was found in cells transfected with the TSH receptors mutated at Asn 77 and Asn 113, as well as with the TSH receptor mutated in all 6 potential glycosylation sites (Fig. 9B). As described above, all three of these mutated TSH receptors had lost their high affinity for TSH binding.

DISCUSSION

Previous studies, involving the chemical or enzymatic removal of carbohydrate moieties, have indicated a role for N-linked oligosaccharide chains in the binding and/or signal transduction properties of several hormone or cytokine membrane receptors including the basic fibroblast growth factor (Feige et al., J. Biol. Chem. 263:14023-14029 (1988)) and interferon-gamma receptors (Fischer et al., J. Biol. Chem. 265:1710-1717 (1990)). At least partial glycosylation of the insulin (Ronnett et al., J. Biol. Chem. 256:4704-4707 (1981)) and EGF receptors (Gamou et al., J. Biochem. 104:388-396 (1988)) appears necessary for ligand binding activity. On the contrary, deglycosylation of the B-adrenergic (Boege et al., J. Biol. Chem. 263:9040-9049 (1988)) and prolactin (Lascols et al., Molec. Cell. Endocrinol. 65:145-155 (1989)) receptors do not influence ligand

binding properties. Contradictory data have been reported regarding the role of glycosylation in LH-receptor activity (Minegishi et al., Proc. Nacl. Acad. Sci. USA 86:1470-1474 (1989); Keinanen, K.P. Biochem. 256:719-724 (1988)).

The recognition sequence for N-linked glycosylation is Asn-X-Thr/Ser (Marshall et al., Annu. Rev. Biochem. 41:673-702 (1972)) and it is well-established that mutagenesis of the Asn amino acid in this consensus sequence is sufficient to prevent transfer of carbohydrate moieties to proteins. This approach makes it possible to investigate the contribution of single carbohydrate moieties in multiglycosylated proteins, and avoids the problems of other methods such as incomplete enzymatic digestion or toxic effects on the protein backbone.

The human TSH receptor amino acid sequence has six potential N-linked oligosaccharide units at asparagine 77, 99, 113, 177, 198 and 302, all placed in the extracellular domain of the protein. By site-directed mutagenesis we replaced asparagine at these potential glycosylated sites with glutamine, an amino acid with a similar polar side chain. A single conservative substitution of an amino acid in an exterior, hydrophilic region of a globular protein is well-tolerated without a major conformational change in tertiary structure (Bowie et al., Science 247:1306-1310 (1990); Argos, P., J. Mol. Biol. 197:331-348 (1987)).

Our data indicate that high affinity hormone binding with subsequent signal transduction is totally dependent on the N-linked glycosylation at Asn 77 in the human TSH receptor. Glycosylation at Asn 113 is less critical, but is still necessary for the full

expression of a functional receptor on the cell surface. The lack of effect of the mutations at Asn 99, 177, 198 and 302 indicate that glycosylation at these sites is not needed for TSH receptor activity.

EXAMPLE V

TSH-LH/CG RECEPTOR EXTRACELLULAR DOMAIN CHIMERAS AS PROBES FOR TSH RECEPTOR FUNCTION

The human TSH receptor (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); Libert, F., et al., Biochem. Biophys. Res. Comm. 165:1250-1255 (1989); Misrahi, M., et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990)) as well as other pituitary (LH and FSH) and placental (CG) glycoprotein hormone receptors (McFarland, K.C., et al., Science 245:494-499 (1989); Loosfelt, H., et al., Science 245:525-528 (1989); Sprengel, R., et al., Mol. Endocrinol. 4:525-530 (1990)), belong to a new sub-group of the guanine nucleotide (G) regulatory protein-coupled receptor family vith very large extracellular domains. With 14 incomplete leucine-rich repeated segments this sub-group of receptors are also members of the leucine-rich glycoprotein family (Takahashi, N., et al., Proc. Natl. Acad. Sci. USA 82:1906-1910 (1985)). There have been no studies to localize in these large extracellular domains the site(s) involved in ligand binding or the region(s) important in signal transduction. For this purpose we performed homologous substitutions in the extracellular domain of the human TSH receptor with segments of the rat lH/CG receptor.

These chimeric TSH-LH/CG receptors reveal that multiple regions in the carboxyl terminal half

(residues 171-418) of the extracellular domain are involved in signal transduction. On the other hand, TSH binding was influenced by homologous substitutions throughout the TSH receptor extracellular domain, suggesting multiple contact points for the hormone. There appears to be considerable tolerance for alteration in the hormone binding region.

The unusually large size of the extracellular domain (418 amino acids) of the TSH receptor (764 amino acids), the very large size (28 kD) of its specific ligand, TSH, as well as cross-linking studies (Buckland, P.R., et al., FEBS Letters 145:245-249 (1982)), make it likely that TSH binds to the extracellular domain of this receptor. Furthermore, hCG binds with high affinity to the extracellular domain of the 1H/CC receptor (Sprengel, R., et al., Mol. Endocrinol. 4:525-530 (1990)). The considerable (30-50%) homology in the extracellular domains of the glycoprotein hormone receptors, together with the presence of 9 conserved extracellular cysteine residues, some of which are thought to form disulfide bonds (Buckland, P.R., et al., FEBS Letters 145:245-249 (1982); Rees Smith, B., et al., The Lancet 427-431 (1974)), suggest a similar three-dimensional structure for the extracellular domains of these receptors, and make them ideal candidates for homologous substitution studies of this region. Thus, in order to conserve the three-dimensional structure of the TSH receptor we constructed 10 chimeric human TSH-rat LH/CG receptor cDNAs (Fig. 10) which were then stably expressed in Chinese hamster ovary (CHO) cells.

METHODS

For chimeric receptor construction we divided the extracellular domain of the human TSH receptor into five regions by restriction endonuclease sites, 3 of which sites were introduced by site-directed mutagenesis. One or more of these regions were replaced with the homologous region of the rat LH/CG receptor (Fig. 10). Pools of stably transfected clones of cells were tested for their ability to bind to TSH and to respond to TSH stimulation in terms of an increase in intracellular cAMP levels.

Construction and functional expression of chimeric TSH-LH/CG receptor cDNAs: Chimeras were constructed using five restriction endonuclease sites in the extracellular domain of the full-length human TSH receptor cDNA in Bluescript (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)) (SnaB I at amino acid 82, Mlu I at amino acid 170, Afl II at amino acid 260, EcoR V at amino acid 360 and Spe I at amino acid 418), and the Sal I site in the multiple cloning site of the vector. Two of the sites in the TSH receptor cDNA (SnaB I and Afl II) were pre-existing restriction sites. The other three sites (Mlu I, EcoR V, Spe I) were chosen by their uniqueness to the plasmid containing the cDNA, and were created by using oligonucleotide-directed mutagenesis (Bio-Rad kit, Richmond, CA) (Kunkel, T.A., Proc. Natl. Acad. Sci. USA 82:488-492 (1985)). These new sites created two conservative amino acid substitutions (Glu→Asp at residue 362, and Ile→Leu at residue 419). The TSH receptor cDNA with the two conserved substitutions was excised with Eco RI and subcloned into the expression

vector pSV2-NEO-ECE (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)).

Rat LH/CG receptor cDNA was synthesized by the polymerase chain reaction (PCR) (Saiki, R.K., et al., Science 239:487-491 (1988)) using as template ~10 ng phage DNA extracted from a rat ovarian library (Clontech, Palo Alto, CA) and two oligonucleotides containing the appropriate restriction sites at their 5'-ends. These restriction sites were then used for substitution of the LH/CG receptor cDNA fragments into TSH receptor cDNA from which the corresponding region had been deleted using the same restriction enzymes. The nucleotide sequences of the PCR-generated fragments of the rat LH/CG receptor, as well as the ligation sites and adjacent nucleotide sequences of the TSH receptor in the chimeric constructs, were determined (Sanger, F., et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)) in full and compared to the previously published sequence data (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); McFarland, K.C., et al., Science 245:494-499 (1989)). Only chimeric cDNAs without any amino acid substitutions were chosen.

The chimeric TSH-LH/CG cDNA was transfected into CHO cells by the calcium-phosphate method (Chen, C., et al., Mol. Cell. Biol. 7:2745-2752 (1987)). Surviving colonies were selected by G418 (400 ug/mL) and pooled for study of their ability to bind to TSH and to respond to TSH with respect to an increase in intracellular cAMP levels.

BH and hCG binding and intracellular cAMP measurements: TSH binding studies were performed as previously described (Chazenbalk, G.D., et al., Endocrinology (in press)) with the exception that TSH

was iodinated with ^{125}I to approximately 80 uCi/ug protein using the Bolton-Hunter reagent. In addition, we tested competition for ^{125}I-TSH binding with hCG as well as TSH. Non-specific ^{125}I-TSH binding was determined in the presence of $10^{-6}M$ TSH and this value was subtracted from total binding to yield specific TSH binding. Measurements of the intracellular cAMP response to hormone stimulation (1 hr at 37°C) was as previously described (Hirayu, H., et al., Molec. Cell. Endocrinol. 42:21-27 (1985)).

RESULTS

The human TSH receptor with two conserved amino acid substitutions (introduced with the three new restriction sites for chimera construction) was identical to the wild-type receptor (Chazenbalk, G.D., et al., Endocrinology (in press)) in terms of its affinity for TSH and in its ability to mediate an increase in intracellular cAMP levels. Of the chimeric receptors in which only a single TSH receptor region was replaced (chimeras TSH-LHR-1 through 5) (Fig. 1 and Table I), chimeras TSH-LHR-1 (substitution of TSH receptor residues 1-82) and TSH-LHR-3 (residues 171-260) lost their high affinity TSH binding site. In contrast, chimeras TSH-LHR-2 (residues 83-170), TSH-LHR-4 (residues 261-360) and TSH-LHR-5 (residues 361-418) bound TSH with high affinity. As with the wild-type TSH receptor (Chazenbalk, G.D., et al., Endocrinology (in press)), high concentrations of hCC ($10^{-6}M$) did not compete for TSH binding to chimeras TSH-LHR-2, TSH-LHR-4 and TSH-LHR-5, indicating that these chimeras retained their specificity for TSH binding. Functionally, as determined by the

intracellular cAMP response to TSH stimulation (Table 3) chimeras TSH-LHR-1 and TSH-LHR-3 were totally inactive, as expected in view of their inability to bind the hormone with high affinity. In contrast, chimeras TSH-LHR-4 and TSH-LHR-5, with high affinities for TSH binding, had blunted cAMP responses to TSH stimulation.

Table 3

Hormone Binding and Function in CHO Cells
Stably Expressing the TSH-LH/CH Chimeras
Depicted in Figure 1
(M - mutant; Wt - wild type)

	Hormone Binding				cAMP Response			
	TSH		hCG		TSH		hCG	
Chimeric Receptor	Kd(nM)	Kd-M / Kd-Wt	Kd(nM)	Kd-M / Kd-Wt+	EC50(nM)	EC50-M / EC50-Wt	EC50(nM)	EC50-M / EC50-Wt#
Wild-Type	0.3	1	n.d.*	n.d.	4.0	1	n.d.	n.d.
TSH-LHR-1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TSH-LHR-2	0.6	2	n.d.	n.d.	6.5	1.6	n.d.	n.d.
TSH-LHR-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TSH-LHR-4	0.2	0.7	n.d.	n.d.	23	5.8	n.d.	n.d.
TSH-LHR-5	0.2	0.7	n.d.	n.d.	20	5.0	n.d.	n.d.
TSH-LHR-6	0.2	0.7	n.d.	n.d.	28	7.0	n.d.	n.d.
TSH-LHR-7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TSH-LHR-8	0.2	0.7	3.4	113	4.8	1.2	20	50
TSH-LHR-9	0.05	0.2	24	800	n.d.	n.d.	n.d.	n.d.
TSH-LHR-10	0.1	0.3	8	267	0.9	0.2	24	60
TSH-LHR-11	n.d.	n.d.	0.03	1	n.d.	n.d.	0.4	1

Table 3: Hormone binding and function in CHO cells stably expressing the TSH-LH/CG chimeras depicted in Fig. 1.

M, mutant; Wt, wild type; *, not detectable.

+, relative to the Kd for hCG binding to TSH-LHR-11.

#, relative to the EC50 for hCG stimulation of TSH-LHR-11.

Each value represents the mean of data obtained with pools of clones from two separate transfections, each transfection measured in duplicate.

The foregoing data suggested that the carboxyl-terminal region of the TSH receptor extracellular domain played a role in signal transduction, and that amino acid residues in two discontinuous regions (1-82 and 171-260) in the TSH receptor extracellular region were important for TSH binding. Surprisingly, however, more extensive substitutions of these regions in chimeras TSH-LHR-8 (residues 1-171) and TSH-LHR-9 (residues 1-260, representing 62% of the extracellular

domain) were associated with high affinity TSH binding (Table 3). Furthermore, these chimeras also lost their complete specificity for TSH and also interacted with hCG, though with a lower affinity for hCG than for TSH. Remarkably, despite their similar, high affinity TSH binding, there was a dichotomy in the functional activity of these two chimeras. Thus, the cellular cAMP response to TSH stimulation with TSH-LHR-8 was similar to that of the wildtype TSH receptor (Table 3). Consistent with the binding data, hCG also increased intracellular cAMP levels in TSH-LHR-8 (400-500% of basal levels with 10^{-7}M hCG). In contrast, chimera TSH-LHR-9 was functionally unresponsive to both TSH and hCG stimulation. This complete dissociation between ligand binding and receptor function in TSH-LHR-8 and TSH-LHR-9 implicates domain C (residues 171-260) (Fig. 10) in the transduction of a signal by the TSH receptor.

Focusing on the carboxyl terminus of the TSH receptor extracellular domain, with chimera TSH-LHR-6 (residues 261-418), a more extensive substitution than TSH-LHR-5 (see above), TSH still bound with high affinity similar to the wild-type receptor (Table 3). However, in contrast to their normal TSH binding, the signal transduction of these mutant receptors was impaired but not abolished (as in TSH-LHR-9). Further substitution of the carboxyl region of the TSH receptor extracellular domain (TSH-LHR-7; residues 171-418) abolished high affinity TSH binding.

Substitutions of both amino-terminal and carboxyl-terminal segments of the TSH receptor in chimera TSH-LHR-10 (residues 1-170 and 361-418) did not alter high affinity TSH binding (Table 3) in accordance with their individual substitutions (TSH-

LHR-8 and TSH-LHR-5). Surprisingly, however, the sensitivity of the cAMP response to TSH stimulation was increased 5-fold relative to the wild-type TSH receptor, and contrasted with the reduced signal transduction with the isolated E domain substitution in chimera TSH-LHR-5.

In the chimeras that bound both TSH and hCG (chimeras TSH-LHR-8, TSH-LHR-9 and TSH-LHR-10), in order to evaluate whether hCG was interacting with the chimeric receptors in the same region as TSH we also tested competition by hCG for ^{125}I-TSH binding. In all three chimeras, hCG competed for *125I-TSH binding but only with low affinity (data not shown).

DISCUSSION

It is well-established that the intracellular, cytoplasmic domains of other members of the C protein-coupled receptor family play an important role in signal transduction (O'Dowd, B.F., et al., J. Biol. Chem. 263:155985-15992 (1988); Strader, C.D., et al., FASEB J. 3:1825-1832 (1989)). The majority of these receptors (which we term group A) interact with very small ligands and lack significant extracellular domains. The present data with the TSH receptor, a member of this receptor family with substantial extracellular domains for large ligands (group B), suggest that the mid-region and carboxyl half of the extracellular domain of the TSH receptor (domains C, D and E) (Fig. 10) play an important role in signal transduction. Domain C appears to be particularly dominant. The data with chimera TSH-LHR-10 also raise the possibility of an interaction between discontinuous amino- and carboxyl-terminal regions of

the receptor extracellular domain (domains A, B and E) in the process of signal transduction.

With respect to hormone binding, substitution of the entire LH/CG receptor extracellular domain for the corresponding region of the TSH receptor (chimera TSH-LHR-11) indicates that the extracellular domain of the glycoprotein hormone receptors is sufficient, and critical, for ligand binding. Nevertheless, it is remarkable that no single domain in the extracellular region of the TSH receptor can be implicated as being a dominant site for high affinity TSH binding. That is, there is at least one chimeric receptor with a substitution for every segment of the TSH receptor extracellular domain (A-E) that binds to TSH with high affinity. It seems likely that the absence of high affinity TSH binding with 3 of the 11 chimeras (TSH-LHR-1, TSH-LHR-3 and TSH-LHR-7) may reflect abnormal folding or instability of the chimeric receptor proteins because substitution of the same regions in other chimeras did not affect TSH binding. These findings suggest that the TSH binding site on the receptor is likely to be discontinuous, with multiple contact points between the two molecules. Unlike with the growth hormone-prolactin hormone family (Cunningham et al., Science 247:1461-1465 (1990); Cunningham et al., Science 243:1330-1336 (1989)), homologous substitutions in a limited number of sites in the TSH receptor is tolerated without significant change in high affinity ligand binding.

The present data provide information not only about the TSH receptor but also about the H/CG receptor. Unlike with the TSH receptor, none of the chimeras studied retained high affinity hCG binding, even though some did interact with this hormone with

relatively low affinity. Therefore, the LH/CG receptor appears to be less tolerant of homologous substitution than the TSH receptor.

The chimeric receptor approach used in this study is a powerful means to define domains important for the unique functions of members of a homologous protein family. However, it does not identify regions that may be involved in a function common to all members of the protein family. For example, the glycoprotein hormones have a common alpha subunit that binds to the extracellular domain of the receptor. This common region would not be identified by the homologous substitution approach used. Indeed, mutagenesis studies of an 8 amino acid region (amino acid residues 38-45) in the human TSH receptor suggests that this region is critical for TSH binding (Wadsworth et al. Science (in press) (1990)). Because homologous substitution of this region did not abolish high affinity TSH binding (present study), it is likely to be a site of interaction with the common alpha subunit of the glycoprotein hormones.

This data provides the first delineation of functional domains in the extracellular component of a member of the newly-discovered group of G protein-coupled receptors with large extracellular domains (sub-group B). The large extracellular domain of the TSH receptor makes this information a practical pre-requisite for future detailed mutagenesis studies to define more precisely the amino acid residues involved in hormone binding and signal transduction.

EXAMPLE VI

HOMOLOGOUS DESENSITIZATION TO THYROTROPIN STIMULATION REQUIRES A CELL-SPECIFIC FACTOR

Hormonal regulation of tissue activity is influenced both by the amount of hormone binding to the tissue and by changes in tissue responsivity to the hormone. Desensitization to continued hormone stimulation is a general phenomenon involving many hormones and hormone-responsive tissues. Desensitization may be homologous (hormone-specific) or heterologous (one hormone inducing desensitization to other hormones). In the case of the thyroid follicular cell, prior thyrotropin (TSH) stimulation leads to a 30-70% decrease in the subsequent cAMP response to TSH stimulation (Rapoport, B., Endocrinology 98:1189-1197 (1976); Shuman, S.J., et al., J. Clin. Invest. 57:1132-1141 (1976)). As with homologous desensitization to a variety of hormones, TSH desensitization appears to involve decreased coupling of its receptor to the adenylate cyclase stimulatory regulatory protein, G_s (Rapoport, B., et al., FEBS Letters 146:23-27 (1982)). Both the functional activity of G_s and the adenylate cyclase catalytic unit remain unaffected (Rapoport, B., et al., FEBS Letters 146:23-27 (1982)).

The mechanism underlying homologous TSH desensitization has not been determined. There is evidence that a putative "desensitization protein" in thyroid cells plays a role in this process (Rapoport, B., et al., J. Biol. Chem. 251:6653-6661 (1976); Filetti, S., et al., J. Biol. Chem. 257:1342-1346 (1982)), as may TSH-mediated ADP-ribosylation of an unknown substrate (Filetti, S., et al., J. Biol. Chem.

256:1072-1075 (1981); Filetti, S., et al., J. Clin. Invest. 68:461-467 (1981)). However, for nearly a decade there has been little progress in this area of investigation, primarily because the TSH receptor has been a poorly-characterized entity, not available for study at a molecular level. Recently, however, the human TSH receptor has been cloned and functionally expressed (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); Misrahi, M., et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990)). The stable expression of this receptor in Chinese hamster ovary (CHO) cells (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)) raises the interesting question of whether, as in thyroid cells, the human TSH receptor, when expressed in non-thyroidal cells, undergoes desensitization to TSH stimulation. The following example indicates that this is not the case, suggesting that a cell-specific factor may be involved in homologous TSH desensitization.

METHODS

Cell cultures: CHO cells stably-transfected with the plasmid p5V2-NEO-ECE-hTSH, and expressing a functional human TSH receptor (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)), were used in these studies. Individual clones were isolated with cloning cylinders and tested for their cAMP response to TSH stimulation (see below). The highest responder was subsequently subcloned further by limiting dilution. Cells from this line (CHO-TSHR) were cultured in 6 or 24 well plates (Costar, Cambridge, MA) in Ham's F12 medium supplemented with

10% fetal calf serum, 100 U/ml penicillin, 40 ug/ml gentamicin, and 2.5 ug/ml amphotericin B. Cryopreserved human thyroid cells were prepared and plated as previously described (Filetti, S., et al., Endocrinology 114:1379-1385 (1984)). Cells were maintained at 37°C in an atmosphere of 95% air-5% CO_2.

Cellular cAMP measurements: Cells were pre-incubated for 16 h in the above medium with or without added TSH (10 mU/ml). The medium was then removed and replaced with fresh medium containing 10 mU/ml TSH and 2 mM isobutyl methylxanthine (IBMX). After 1 h at 37°C, cellular cAMP was extracted with 95% ethanol and measured by radioimmunoassay, as previously described (Hirayu, H., et al., Molec. Cell. Endocrinol. 42:21-27 (1985)).

Radiolabeled TSH binding: Highly-purified bTSH (approximately 30 U/mg protein) was radiolabeled with Na-^{125}I (Amersham, Arlington Heights, IL) (Goldfine, I.D., et al., Endocrinology 95:1228-1233 (1974)). The CHO-TSHR cells were rinsed three times with a modified Hank's buffer without NaCl, with isotonicity maintained with 280 mM sucrose (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)). The cells were then incubated under conditions indicated In the text in the same buffer, supplemented with 0.25% bovine serum albumin (BSA), approximately 10^{-12}M ^{125}I-bTSH, and the indicated amount of unlabeled bTSH (Thytropar, Kankakee, IL) or other agents. At the end of the incubation period the cells were rapidly rinsed three times with the same buffer (4°C) lacking TSH, solubilized with 1 ml 1 N NaOH, and radioactivity was measured in a gamma counter. Protein concentrations were measured in the same extract by the method of Bradford (Bradford, M.M., Anal. Biochem. 72:248-254

(1976)) using BSA standards. Non-specific ^{125}I-TSH binding was measured in the presence of 10^{-6} TSH, and this value was subtracted from total binding to yield specific TSH binding values. The range of specific TSH binding in different experiments was 5-12% of total counts added. Non-specific binding was approximately 1% of total counts added. Control studies included the binding of ^{125}I-TSH to plastic dishes without cells, and to untransfected CHO cells not expressing the TSH receptor. Indeed, in preliminary studies, the low affinity, high capacity binding of ^{125}I-TSH to plastic, as previously observed (Yamamoto, M., et al., Endocrinology 103:2011-2019 (1978)), obscured the detection of high affinity, specific binding to CHO-TSHR cells. This difficulty was overcome lay performing the TSH binding studies only when the dishes of cells were completely confluent, and using a minimal volume of buffer to avoid contact with the walls of the dishes. In retrospect, the likely explanation for our previous inability to detect a high affinity TSH binding site in primary cultures of human thyroid cells is that human thyroid cells, unlike CHO-TSHR (present study) and FRTL5 cells (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)), do not proliferate, and therefore some plastic surface of the dish is always exposed.

RESULTS

The Chinese hamster ovary cell line CHO-TSHR, expressing a functional recombinant human TSH receptor, was established following cloning and subcloning by limiting dilution. In preliminary

experiments, maximal TSH stimulation (10 mU/ml) increased CHO-TSHR intracellular cAMP levels 8-10-fold. Similarly to human thyroid cells (Rapoport, B., et al., Metabolism 31:1159-1167 (1982)), maximal stimulation of CHO-TSHR cells was attained after 30-60 min of exposure. Surprisingly, however, when CHO-TSHR cells were pre-incubated for 12-16 h with TSH (10 mU/ml), there was no diminution in the subsequent cAMP response to a 1 h pulse of TSH stimulation (Fig. 11). This time period of continued exposure to TSH is more than sufficient to induce maximal TSH desensitization in dog, human and rat thyroid cells. In contrast, as previously reported (Filetti, S., et al., J. Biol. Chem. 257:1342-1346 (1982)), pretreatment for 16 h of human thyroid cell monolayer cultures with TSH decreased (by 65% in the experiment shown) the subsequent cAMP response to TSH stimulation. These data indicate that, unlike the human TSH receptor in human thyroid cells, this receptor in CHO-TSHR cells does not undergo desensitization to TSH stimulation.

In view of this functional difference of the human TSH receptor when stably expressed in non-thyroidal eukaryotic cells, we studied the characteristics of TSH binding to this receptor. In contrast to many prior experiments with human thyroid cell monolayer cultures (Yamamoto, M., et al., Endocrinology 103:2011-2019 (1978)), specific TSH binding was readily detected in CHO-TSHR cells, with a time course similar to that observed with FRTL5 rat thyroid cells (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)) (Fig. 12). Based on these data, subsequent ^{125}I-TSH binding studies were performed for 2 h at 37°C. Competition inhibition by unlabeled TSH of ^{125}I-TSH binding to CHO-TSHR cells revealed a

biphasic curve, suggesting two types of binding sites, one of higher and one of lower affinity (Fig. 13). In contrast, wild-type, untransfected CHO cells exhibited only a single binding site of low affinity (Fig. 13). Scatchard plot analysis of the specific TSH binding to the CHO-TSHR cells revealed a high affinity site of $1.8 \pm 0.4 \times 10^9$ M^{-1} and a low affinity site of $1.4 \pm 0.3 \times 10^7$ M^{-1} (Fig. 14). Based on Avogadro's number, there are approximately 10^5 TSH receptors per CHO-TSHR cell. As previously reported for FRTL5 cells (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)), when ^{125}I-TSH binding was conducted in isotonic Hank's buffer containing NaCl Instead of sucrose, the number of TSH binding sites was reduced by approximately 10-fold, without any alteration in the affinity of TSH binding (data not shown).

Previous studies with FRTL5 rat (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)) and pig (Takasu, N., et al., Eur. J. Biochem. 90:131-138 (1978)) thyroid cells indicate that prolonged (16-24 h) exposure of these cells to TSH reduces the number cf TSH receptors available for TSH binding. This TSH receptor "down-regulation" cannot be the basis for TSH desensitization in FRTL5 thyroid cells because the latter is evident much earlier, within 2 h of TSH stimulation (Hirayu, H., et al., Molec. Cell. Endocrinol. 42:21-27 (1985)). Nevertheless, it was of interest to determine whether or not TSH receptor "down-regulation" occurs in CHO-TSHR cells expressing recombinant human TSH receptor. Prior exposure of these cells to 10 mU/ml bTSH for periods up to 24 h did not significantly reduce the extent of ^{125}I-TSH binding (Fig. 15). Because of the report that cAMP analogs, as well as TSH, could lead to TSH receptor

down-regulation in FRTL5 cells (Avivi, A., et al., Science 214:1237-1239 (1981)), we examined the effect of pretreatment with dBcAMP (1 mM) on TSH binding to CHO-TSHR cells. No effect was observed (Fig. 15).

DISCUSSION

The primary, and surprising, finding resulting from this study is that prior TSH stimulation does not lead to functional desensitization of the TSH receptor when expressed in a non-thyroidal eukaryotic cell line. As discussed above, there has been a hiatus of about a decade in advances relating to the mechanism underlying functional TSH desensitization. Previous evidence led to the suggestion that a functional alteration in the TSH receptor, possibly related to a putative "desensitization protein" (Rapoport, B., et al., J. Biol. Chem. 251:6653-6661 (1976); Filetti, S., et al., J. Biol. Chem. 257:1342-1346 (1982)), reduced the coupling of the receptor to the G_s component of the adenylate cyclase regulatory system (Rapoport, B., et al., FEBS Letters 146:23-27 (1982)). The molecular cloning of the TSH receptor may now permit further elucidation of this mechanism, in particular the identification of this putative desensitization protein, which appears to be absent in CHO cells and may therefore be thyroid-specific. The TSH receptor belongs to a family of G protein-coupled receptors with seven transmembrane domains. One member of this family is rhodopsin. Recently, a regulatory protein, termed arrestin, has been identified that inhibits rhodopsin functional activity (Kühn, H., et al., FEBS Letters 176:473-478 (1984); Wilden, U., et al., Proc. Natl. Acad. Sci. USA 83:1174-1178 (1986)). A similar

mechanism has been suggested for beta-adrenergic desensitization (Enzmann, D.R., et al., J. Computer Assisted Tomography 3:815-819 (1979)). Conceivably, there could be a thyroid cell-specific arrestin variant for the TSH receptor.

The molecular cloning of the human TSH receptor (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); Libert, F., et al., Biochem. Biophys. Res. Comm. 165:1250-1255 (1989); Misrahi, M., et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990)) will facilitate studies on the interaction between TSH and its receptor. Libert et al. (Libert, F., et al., Biochem. Biophys. Res. Comm. 165:1250-1255 (1989)) and Misrahi et al. (Misrahi, M., et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990)) demonstrated TSH binding to cultured non-thyroidal cells transiently transfected with the human TSH receptor cDNA. However, the instability of a transient expression system makes it difficult to perform experiments requiring observations at different time points. In contrast, the stable cell line CHO-TSHR, obtained by limiting dilution and not available at the time of our initial report of human TSH receptor expression (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)), has enabled the inventors to study whether or not the TSH receptor in this cell undergoes down-regulation. Characterization of the TSH receptor in CHO-TSHR cells reveals both high affinity (Ka $1.8 \pm 0.4 \times 10^9\ M^{-1}$) and low affinity (Ka $1.4 \pm 0.3 \times 10^7 M^{-1}$) TSH binding sites. These values are remarkably similar to the initial reports for the non-recombinant TSH receptor in human thyroid membranes (Bashford, C.L., et al., Biochem. J. 146:473-479 (1975)) and FRTL5 cells (Tramontano, D.,

et al., Endocrinology 118:1945-1951 (1986)). Subsequent reports on the kinetics of TSH binding to human thyroid tissue are too numerous to list. It is interesting that there are approximately 10^5 TSH receptors per CHO-TSHR cell. This is 10-100-fold greater than the number of TSH receptors estimated to be present on human thyroid cells (Rees Smith, B., et al., Endocr. Rev. 9:106-121 (1988)), and (in addition to non-confluence of the cells in the culture dishes; see Methods) may explain our previous inability to observe specific, high affinity binding to living human thyroid cells (Yamamoto, M., et al., Endocrinology 103:2011-2019 (1978)).

While most investigators have previously assumed that the high affinity TSH binding site is the one of most physiological relevance, the significance of the low affinity TSH binding site in thyroid tissue has been uncertain. The present observation of a high affinity TSH binding site only in CHO cells expressing the TSH receptor, and not in otherwise identical non-transfected CHO cells, suggests that the low affinity binding site in thyroid tissue is unrelated to the TSH receptor. Support for this conclusion is the observation of a low affinity TSH binding site on plastic culture dishes (Yamamoto, M., et al., Endocrinology 103:2011-2019 (1978)) with an affinity similar to that on wild-type CHO cells.

It must be emphasized that although the lack of TSH receptor down-regulation in CHO-TSHR cells is of mechanistic interest in itself, it cannot explain the lack of functional desensitization. In general, in many ligand-receptor systems the phenomenon of receptor down-regulation is only observed after hormone desensitization is already established, and

cannot therefore be the cause of the desensitization. Specifically, in the case of thyroid cells, functional desensitization is observed within 2 h of TSH stimulation, and is near maximal within 4-6 h in dog, human and FRTL5 rat thyroid cells (Rapoport, B., et al., J. Biol. Chem. 251:6653-6661 (1976); Filetti, S., et al., J. Biol. Chem. 256:1072-1075 (1981); Hirayu, H., et al., Molec. Cell. Endocrinol. 42:21-27 (1985)). In contrast, in those studies in which it has been observed, TSH receptor down-regulation occurred only after 16 h (Takasu, N., et al., Eur. J. Biochem. 90:131-138 (1978)) and 24 h (Tramontano, D., et al., Endocrinology 118:1945-1951 (1986)) of TSH stimulation in pig and FRTL5 thyroid cells, respectively.

EXAMPLE VII

The TSH receptor and the receptors for the other pituitary and placental glycoprotein hormones, LH/CG (McFarland, K.C. et al., Science 245:494-499 (1989); Loosfelt, H. et al., Science 245:525-528 (1989)) and FSK (Sprengel, R. et al., Mol. Endocrinol. 4:525-530 (1990)), differ from other members of this receptor family by virtue of their large extracellular domains, which are consistent with the much larger size of their respective hormones. However, studies with the small ligand members of the receptor family suggest that the cytoplasmic domains play an important role in signal transduction by interacting with G proteins. The specific cytoplasmic regions involved in signal transduction have been partially characterized in the α- and β-receptor (Kobilka, B.K. et al., Science 240:1310-1316 (1988); Strader, C.D. et al., J. Biol. Chem. 262:16439-16443 (1987); O'Dowd, B.F. et al., J.

Biol. Chem. 263:15985-15992 (1988)) and in rhodopsin (Franke, R.R. et al., J. Biol. Chem. 263:2119-2122 (1988)). As yet there are no reports on the function of the corresponding regions in the glycoprotein hormone receptors. There is little homology between the amino acid sequence of the intracytoplasmic components of the TSN receptor with the corresponding regions of the small ligand-binding members of the family. To address the question of which cytoplasmic regions in the TSH receptor are involved in the generation of cAMP we have performed substitutional and deletional mutations in these regions of the receptor. Our results show significant differences, as well as some similarities, with the data reported for the small-ligand members of this receptor family.

METHODS

Site-directed mutagenesis and functional expression of the human TSH receptor:

Because we had no preconception as to the regions of importance for signal transduction in the TSH receptor, we decided on a generalized approach to mutagenesis rather than targeting single amino acid residues. Because of the potential importance of charged residues (Clu, Asp, Lys, Arg and His) we substituted these with neutral hydrophilic residues. Ser and Thr, as potential phosphorylation sites were substituted with Gly or Ala. Tyr, a more hydrophobic, potentially phosphorylated residue was substituted with Phe. Cys was substituted with structurally similar Ser. The carboxyl terminus of the receptor was also truncated by the insertion of stop codons. Mutant TSH receptor cDNA was prepared by the method of

Kunkel (Kunkel, T.A., Proc. Natl. Acad. Sci. USA 82:488-492 (1985)) using the Bio-Rad mutagenesis Muta-Gene Phagemid vitro mutagenesis kit according to the protocol of the manufacturer. Oligonucleotides were synthesized by the Molecular Biology Resource Center, University of California, San Francisco. Single-stranded DNA was prepared from the full-length human TSH receptor in Bluescript (Nagayama, Y. et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)) using the helper phage R408 (Stratagene, San Diego, CA). Confirmation of the mutagenized region, and adjacent regions, was obtained by nucleotide sequencing (Sanger. F. et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)) in all clones. The mutagenized TSH receptor cDNA was excised with Eco RI and subcloned into the Eco RI site of the vector pSV2-neo-ECE (Nagayama, Y. et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)). The plasmid constructs containing the mutated TSH receptor were stably transfected (Chen, C. et al., Mol. Cell. Biol. 7:2745-2752 (1987)) into CHO-K1 cells. Cells were grown in Ham's F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 40 ug/ml gentamicin and 2.5 ug/ml amphotericin B. For selection of stably transfected cells, this medium was supplemented with G418 (400 ug/ml). Surviving clones were pooled to generate a series of non-clonal cell lines expressing mutant TSH receptors.

TSH Binding. Highly purified bTSH (5 ug, 30 U/mg protein) was radiolabeled with ^{125}I to a specific activity of approximately 80 uCi/ug protein using the Bolton Hunter reagent (4-00 Ci/mmol; New England Nuclear, Boston, MA) according to the protocol of the

manufacturer, followed by Sephadex G100 chromatography. TSH receptor-expressing CHO cell lines were grown to confluence in 2 cm^2 diameter dishes. Cells were incubated for 2 hrs at 37°C in 0.25 ml modified Hank's buffer without NaCl with isotonicity maintained with 280 mM sucrose, supplemented with 0.25% bovine serum albumin. ^{125}I bTSH (- 1.5 x 10^4 cpm) was included in this buffer together with varying concentrations of unlabeled bTSH (Sigma Chemical Co., St. Louis), as previously described (Chazenbalk, G.D. et al., Endocrinology (in press) (1990)). At the end of the incubation period the cells were rapidly rinsed three times with the same buffer (ice-cold) without TSH, solubilized with 1 ml 1 N NaOH and radioactivity was measured in a gamma counter. Specific TSH binding was defined as the difference between the total binding of ^{125}I-TSH in the absence and presence of $10^{-6}M$ TSH. Non-specific binding was <10% of total binding. Control studies included the binding of ^{125}I-TSH to CHO cells transfected with the wild-type TSH receptor and CHO cells transfected with the vector not containing TSH receptor cDNA.

Cellular cAMP measurements. Transfected cells in 2 cm^2 dishes were incubated in fresh Ham's F12 medium (see above) containing the indicated concentrations of TSH and 2 mM isobutylmethylxanthine (IBMX). After 1h at 37°C, cellular cAMP was extracted with 95% ethanol and measured by radioimmunoassay as previously described (Hirayu, H. et al., Molec. Cell. Endocrinol. 42:21-27 (1985)).

RESULTS

Mutations in the first cytoplasmic loop. Seven amino acid substitutions were made in the first cytoplasmic loop (between residues 441-450) (Fig. 16A). Ligand binding studies showed that the affinity of TSH for this mutant receptor (MUT1-TSH-R) was similar to that of the wild type receptor (Kd ~2 x 10^{-10}M (Fig. 16B and Table 3). Despite normal TSH binding, TSH did not increase cAMP levels in MUT1-TSH-R expressing cells (Fig. 16C).

Mutations in the second cytoplasmic loop. Because of the greater length of the second intracytoplasmic loop, two separate TSH receptor mutants were created. Seven amino acid substitutions were made in the carboxyl terminal region of this loop (residues 528-537; MUT2-TSH-R) (Fig. 17A). The affinity of TSH binding to MUT2-TSH-R was very similar to the wild type receptor (Kd 2 x 10^{-10}M) (Fig. 17B and Table 3). TSH stimulation did not increase intracellular cAMP levels in this mutant receptor (Fig. 17C).

Four residues were mutated in the amino terminus of the second intracytoplasmic loop (residues 518-524; MUT3-TSH-R) (Fig. 17A). This mutant receptor was characterized by a Iou affinity for TSH binding (Kd ~ 4 x 10^{-8} M). As a negative control, TSH binding to CH0 cells transfected with the vector alone (pSV2-neo) revealed a Kd of approximately 9 x 10^{-8}M (Fig. 17B). As with MUT2-TSH-R, TSH stimulation of cells expressing MUT3-TSH-R did not increase intracellular cAMP levels (Fig. 17C).

Mutations in the third cytoplasmic loop. As with the second intracytoplasmic loop, the third loop was divided into two regions for study. Seven amino acid substitutions were made in the carboxyl terminus of this loop (residues 617-625; MUT4-TSH-R) (Fig. 18A). This mutant receptor had an affinity for TSH about 10-fold lower than the wild-type TSH receptor (Fig. 18B and Table 3). TSH was without effect on cAMP levels in cells expressing MUT4-TSH-R (Fig. 18C).

Five residues were mutated in the amino terminal region area of the third cytoplasmic loop (603-613; MUT5-TSH-R) (Fig. 18A). The affinity of TSH binding to this mutant was the same as that for the wild type TSH receptor (Kd of approximately 2 x 10^{-10}M) (Fig. 18B, Table 3). TSH stimulation of cells expressing MUT5-TSH-R led to an increase in cAMP values similar to that with the wild-type TSH receptor (Fig. 18C).

Mutations in the cytoplasmic tail. Two TSN receptor mutants with carboxyl terminus truncations were generated by the introduction of stop codons (Fig. 19A). In TSH receptor mutant MUT6-TSH-R the cytoplasmic tail (amino acids 683 to 764) was completely deleted. In TSH receptor mutant MUT8-TSH-R the carboxyl terminal two-thirds of the cytoplasmic tail that has no homology with the LH/CC receptor was deleted (amino acids 709-764). The remaining amino terminus of the cytoplasmic tail of this mutant is homologous to the LH/CC receptor (Nagayama, Y. et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)). The affinity of TSH binding to MUT6-TSH-R (Kd 2 x 10^{-8}M) was lower than that of TSH to the wild-type receptor while that of MUT8-TSH-R (Kd ~ 2 x 10^{-10}M) was similar to that of the wild-type receptor (Fig. 19B,

Table 3). MUT6-TSH-R was completely unresponsive to TSH stimulation in terms of intracellular cAMP generation whereas the response in the partial deletion (MUT8-TSH-R) was similar to that with the wild-type TSH receptor (Fig. 19C, Table 3).

Because of the potential importance of a cluster of basic amino acids, as well as the presence of a tyrosine residue, in the mid-region of the cytoplasmic tail (residues 699-707), another TSH receptor mutant (MUT7-TSH-R) was created with 5 amino acid substitutions in this region (Fig. 19A). The affinity of this mutant for TSH was similar to that of the wild-type receptor (Fig. 19B). While cAMP levels in MUT7-TSH-R were increase by TSH stimulation, in three separate pools of stably transfected cells the extent of this cAMP response was less than with the wild-type receptor, but the EC50 appeared to be similar to the wild type receptor (Fig. 19C, Table 3).

DISCUSSION

The family of G protein-coupled receptors with seven transmembrane domains appears to be divisible into two sub-groups. One sub-group of receptors (which we term A) contains essentially no extracellular region and interacts with very small ligands. Included in this sub-group are the α and β adrenergic, muscarinic cholinergic, dopamine, serotonin, neuropeptide Y, substance K receptors and rhodopsin (Johnson, G.L. et al., Endocr. Rev. 10:317-331 (1989)). In contrast, a second sub-group of receptors has been identified in this family (sub-group B) which contains a large (360-418 amino acid) extracellular domain, and which interacts with the

large pituitary/placental glycoprotein hormones, TSH LH/CC and FSH (Nagayama, Y. et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); Libert, F. et al., Biochem. Biophys. Res. Comm. 165:1250-1255 (1989); Misrahi, M. et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990); McFarland, K.C. et al., Science 245:494-499 (1989); Loosfelt, H. et al., Science 245:525-528 (1989); Sprengel, R. et al., Mol. Endocrinol. 4:525-530 (1990)). It is possible that the receptors for other large or intermediate-sized hormones, such as parathyroid hormone, will belong to sub-group B.

All receptors in the family contain three cytoplasmic loops and a cytoplasmic tail. In terms of signal transduction, which involves the interaction between these receptors and the guanine nucleotide (C) regulatory proteins, mutagenesis studies with sub-group A (adrenergic and rhodopsin) receptors (Strader, C.D. et al., J. Biol. Chem. 262:16439-16443 (1987); O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988); Franke, R.R. et al., J. Biol. Chem. 263:2119-2122 (1988)) implicate the cytoplasmic domains in this process. In particular, the carboxyl terminus of the third cytoplasmic loop and the amino terminus of the cytoplasmic tail appear to play an important role in signal transduction. At present, there have been no reports on this subject in regard to the glycoprotein hormone (sub-group B) receptors. It is of value to consider the data obtained for the TSH receptor with those reported for the sub-group A receptors because there are both differences and similarities. The importance of the first cytoplasmic loop in the activation of adenylate cyclase has not been definitively determined in a sub-group A receptor

because of the deleterious effects of mutations in this region of the molecule on the level of receptor expression (O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988); Dixon, R.A.F. et al., EMBO J. 6:3269-3275 (1989)). In contrast, mutagenesis of 7 of the 10 amino acids in the first cytoplasmic loop of the TSH receptor (MUT1-TSH-R) had no effect on ligand (TSH) binding but totally abolished the ability of the receptor to increase intracellular cAMP levels. These data strongly suggest that the first cytoplasmic loop, at least in the TSH receptor is important in the coupling of the receptor to Gs.

We have determined that the carboxyl terminal region of the second cytoplasmic loop of the TSH receptor is essential for functionally normal receptor G protein interaction. Substitutions in the carboxyl-terminal (MUT2-TSH-R) region generated a receptor with an unaltered affinity for TSH yet stimulation of intracellular cAMP levels was abolished, indicating a loss of signal transduction. These data differ significantly with those for the β-adrenergic receptors in which the carboxyl-terminal region of the second cytoplasmic domain was not found to be involved in G protein interaction. Similar to our data with the TSH receptor, the importance of the amino-terminus of this loop could not be determined in the mutated β-adrenergic receptor because it was incapable of ligand binding (O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988)). A proline in the mid-region of the second cytoplasmic loop of the β2-adrenergic receptor that was found to be of importance in signal transduction (O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988)) is not present in this region of the human TSH receptor.

In regard to the third cytoplasmic loop, the data obtained with the TSH receptor are more consistent with the data for the sub-group A receptors. Thus, a mutant rhodopsin with a single amino acid substitution in the carboxyl terminus of this cytoplasmic loop led to failure of signal transduction (Franke, R.R. et al., J. Biol. Chem. 263:2119-2122 (1988)). More extensive mutagenesis of the β-adrenergic receptor had a similar functional effect (O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988)). Similarly, mutagenesis of the carboxyl terminus of the third cytoplasmic loop of the TSH receptor (MUT4-TSHR) dramatically interfered with signal transduction. In addition, in this mutant the affinity of TSH binding was approximately one order of magnitude lower than that of the wild-type TSH receptor. Extensive mutagenesis of the amino terminus of the third cytoplasmic loop of the TSH receptor was without effect on either the affinity of TSH binding or on the ability of TSH to increase intracellular cAMP levels. These data suggest that the amino-terminal segment of the third cytoplasmic loop of the TSH receptor is not involved in coupling to Gs. Contradictory data have been reported as to the functional importance of the amino terminus of the third cytoplasmic loop of the β-adrenergic receptor (O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988); Strader, C.D. et al., J. Biol. Chem. 262:16439-16443 (1987)).

Deletion of the entire cytoplasmic tail of the TSH receptor (MUT6-TSH-R) greatly reduced the affinity of TSH binding to the receptor by about two orders of magnitude making it difficult to interpret the lack of a cAMP response to TSH stimulation in cells bearing these mutant receptors. In contrast, deletion of the

carboxyl terminal 56 amino acids of the TSH receptor (MUT8-TSH-R) had no effect on the affinity of the receptor for TSH or on the ability of the receptor to increase intracellular cAMP levels. It is noteworthy that the carboxyl terminal region deleted in MUT8-TSH-R has no homology with the LH receptor in contrast to the remaining amino terminal segment of the cytoplasmic tail. Substitution mutagenesis of a number of potentially important positively charged residues and a tyrosine in the cytoplasmic tail (between residues 699-707) had no effect on the affinity of TSH binding but did appear to diminish the cAMP responsiveness to TSH stimulation. Therefore, unlike with data obtained with the β-adrenergic receptor (O'Dowd, B.F. et al., J. Biol. Chem. 263:15985-15992 (1988); Dixon, R.A.F. et al., EMBO J. 6:3269-3275 (1989)) and rhodopsin (Strader, C.D. et al., J. Biol. Chem. 262:16439-16443 (1987)), our studies do not provide definitive evidence to support the importance of the amino-terminal region of the cytoplasmic tail (residues 683-709) in signal transduction. However, we can conclude that the non-conserved carboxyl two-thirds of the cytoplasmic tail of the human TSH has no influence on this process. In summary, this study provides the first information on the importance of several regions of the cytoplasmic domains of the TSH receptor, a member of a new sub-group of G protein-coupled receptors, on signal transduction in response to hormone stimulation. These data reveal interesting differences and similarities with the sub-group of receptors lacking significant extracellular domains, as exemplified by the β-adrenergic receptor. The most important regions of the TSH receptor for signal transduction appear to

be the relatively small first cytoplasmic loop (residues 441-450), the carboxyl-terminal regions of the second cytoplasmic loop (residues 528-537) and the carboxyl-terminal (amino acids 603-614) of the third cytoplasmic loop.

EXAMPLE VIII

Antibodies against the TSH receptor are present in the serum of many patients with autoimmune thyroid disease, more so in Graves' disease than in Hashimoto's thyroiditis (Rees Smith et al., Endocr Rev. 9:106-121 (1988)). These autoantibodies are capable of interacting with the TSH receptor, and of either mimicking the action of TSH in leading to thyroid hyperfunction, or of blocking TSH action with consequent hypothyroidism. There are, at present, two basic types of assays for detecting anti-TSH receptor antibodies. One, by inducing a biological response such as cAMP generation in intact thyroid cells, measures the antibodies that react with the TSH receptor in a stimulatory manner (Hinds et al., J Clin Endocrinol Metab. 52:1204-1210 (1981); Kasagi et al., J Clin Endocrinol Metab. 54:108-114 (1982)). The other type of assay is not a stimulatory bioassay, but instead measures antibodies that interact with the receptor and compete for TSH binding (Rees Smith et al., The Lancet 427-431 (1974)). This TSH binding inhibition (TBI) assay detects TSH receptor antibodies of both the stimulatory and the inhibitory variety without discriminating between the two (Rees Smith et al., Endocr Rev. 9:106-121 (1988)). Both types of antibodies may be present in the same patient (Zakarija et al., J Clin Invest. 72:1352-1356 (1983)).

The molecular cloning and expression of the human TSH receptor has recently been achieved (Nagayama et al., Biochem Biophys Res Comm. 165:1184-1190 (1989); Libert et al., Biochem Biophys Res Comm. 165:1250-1255 (1989); Misrahi et al., Biochem Biophys Res Comm. 166:394-403 (1990)). The availability of Chinese hamster ovary (CHO) cells stably transfected with the human TSH receptor cDNA and expressing a functional TSH receptor (Nagayama et al., Biochem Biophys Res Comm. 165:1184-1190 (1989)) now provides the opportunity for detecting anti-TSH receptor autoantibodies with this recombinant antigen. The present example describes the use of these cells in a TSH binding inhibition (TBI) assay.

METHODS

Cell cultures: Three types of cells were used in this study; CHO cells expressing a functional human TSH receptor (CHO-TSHR) (Nagayama et al., Biochem Biophys Res Comm. 165:1184-1190 (1989); Chazenbalk et al., Endocrinology (in press) (1990)) and FRTL5 rat thyroid cells (Ambesi-Impiombato et al., Proc Natl Acad Sci USA 77:3455-3459 (1980)) and primary cultures of human thyroid cells. Cells were cultured in 24 well plates (Costar, Cambridge, MA). CHO-TSHR cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 40 μg/ml gentamycin, and 2.5 μg/ml fungizone. FRTL5 cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum, penicillin (125 U/ml), gentamycin (40 μg/ml), amphotericin B (2.5 μg/ml) and a mixture of three hormones; bTSH (5 mU/ml), transferrin (5 μg/ml) and insulin (10 mU/ml). At confluence, this medium is

replaced for 5-7 d with the same medium with the exception that TSH is omitted. Human thyroid cells were prepared by collagenase digestion from thyroid tissue discarded after surgery for appropriate medical reasons (Rapoport et al., Metabolism 31:1159-1167 (1982)). One or two days before experiment cells were thawed and plated in M199 medium containing 10% fetal calf serum, 2mM glutamine and non-essential amino acids. All cells were maintained at 37°C in an atmosphere of 95% air-5% CO_2.

Patients: Sera from 228 patients with thyroid disease and 35 normal controls were studied. The diagnosis of Graves' disease with hyperthyroidism was made by conventional clinical criteria, including an elevated free T4 index in association with an increased thyroidal radioactive iodine uptake. The diagnosis of Hashimoto's thyroiditis was made on the basis of elevated anti-microsomal (thyroid peroxidase) or anti-thyroglobulin antibody titers in euthyroid subjects with goiter or in hypothyroid patients with or without goiter. In patients with goiter the diagnosis of Hashimoto's thyroiditis was confirmed by fine needle aspiration biopsy.

Radiolabeled TSH binding: Highly-purified bTSH (approximately 30 U/mg protein) was radiolabeled with Na-^{125}I (Amersham, Arlington Heights, IL) (Goldfine et al., Endocrinology 95:1228-1233 (1974)). In some experiments radiolabeling with ^{125}I was preformed with the Bolton-Hunter reagent (Amersham, Milan, Italy) according to the protocol of the manufacturer. Free iodide was removed by Sephadex G100 chromatography. The specific activity of the radiolabeled TSH was 80-150 μCi/μg protein. CHO-TSHR or FRTL5 cells were rinsed three times with a modified Hank's buffer

without NaCl, and with isotonicity maintained with 280 mM sucrose (Tramontano et al., Endocrinology 118:1945-1951 (1986)). The cells were then incubated under conditions indicated in the text in the same buffer, supplemented with 0.25% bovine serum albumin (BSA), approximately 10^{-12}M ^{125}I-bTSH, and the indicated amount of unlabeled bTSH (Thytropar, Kankakee, IL) or IgG preparation. At the end of the incubation period, the cells were rapidly rinsed three times with the same buffer (4°C) lacking additives, solubilized with 1 ml 1 N NaOH, and radioactivity was measured in a gamma counter. Non-specific ^{125}I-TSH binding was measured in the presence of 10^{-6}M TSH, and this value was subtracted to yield specific TSH binding values. The range of specific TSH binding in different experiments was 8-15% of total counts added. Non-specific binding was approximately 1%. IgG from patients' sera were prepared using either DEAE-Affygel blue column chromatography (Bio-Rad Laboratories, Milan, Italy). IgG was concentrated by ultrafiltration (Centriflo cones, Amicon Corp., Danvers, MA) to 30-40 mg/ml (Extinction at 1% = 13.5; 280 nm). Crude IgG was prepared by mixing 0.5 ml of serum with 1.5 ml of 20% polyethylene glycol 4000 (PEG). After centrifugation (20 min at 3000 x g) the pellet was dissolved in 0.6 ml of KRS binding buffer (see above). Aliquots (0.3 ml) were added to duplicate wells for determination of TSH binding inhibition (TBI) activity. The TBI assay was performed by modifying the conditions previously reported for TSH binding to these cells (Chayenbalk et al., Endocrinology (in press) (1990)). In brief, after preincubation of the cells for 2 h at 37°C with 0.3 ml of PEG-prepared IgG the cells were rinsed twice

with 1 ml of ice-cold KRS buffer followed by a second incubation (1 - 1.5 h at 37°C) in 0.3 ml pre-warmed KRS buffer containing approximately 10*4 cpm of ^{125}I-TSH. The cells were then rinsed three times with 1 ml of ice-cold KRS. After the solubilization of cells by the addition of 0.5 ml of 1 N NaOH to the wells, 0.4 ml was counted for radioactivity in a gamma counter.

In the study of TSH binding inhibition (TBI) activity in the sera of patients with thyroid disorders, TBI values were normalized to an index of 1.0, with this number representing 100% of specific ^{125}I-TSH binding.

Thyroid stimulating immunoglobulin bioassay: This assay was performed as previously described in detail using primary monolayer cultures of human thyroid cells (Rapoport et al., J Clin Endocrinol Metab. 58:332-338 (1984)). Data are expressed, however, as % of basal (unstimulated) cAMP values instead of TSH uU equivalents.

Solubilized porcine TSH receptor assay: The TRAK radioreceptor assay for measuring TSH receptor antibodies (Henning, Berlin) was used according to the protocol of the manufacturer (15 min preincubation with serum at room temperature prior to the addition of ^{125}I-TSH for 1 h at 37°C). In some experiments highly-pufified IgG was used instead of whole serum.

RESULTS

Purified IgG containing potent thyroid stimulating immunoglobulin (TSI) bioactivity competed for radiolabeled TSH binding to recombinant TSH receptors expressed on CHO cells in parallel to inhibition by unlabeled TSH (Fig. 20). Highly-

purified IgG from a normal individual competed weakly for TSH binding, and only at the highest IgG concentration tested (10 mg/ml) (Fig. 20). Using the same IgG preparation with potent TSI activity, the recombinant TSH receptor system was approximately 3-fold more sensitive than FRTL5 rat thyroid cells, and 10-fold more sensitive than the solublized porcine TSH receptor (TRAK) assay (Fig. 21). These data indicate the feasability for using the recombinant TSH receptor stably expressed in CHO cells for detecting TSH binding inhibition (TBI) activity in the sera of patients with autoimmune thyroid disease.

Next examined was the behaviour of the recombinant TSH receptor assay with a series of sera from patients with thyroid dysfunction, primarily Graves' disease. For the purpose of establishing a practical assay, instead of highly-purified (DEAE-Sephadex) IgG, we utilized polyethylene glycol (PEG)-precipitated IgG (Kasagi et al., J Clin Endocrinol Metab. 62:855-862 (1986)). To avoid the interference of the PEG on the radiolabeled TSH, we performed a variation of the TBII residual binding assay of Borges et al. (Borges et al., J Clin Endocrinol Metab. 54:552-558 (1982)). The principle of this two-step, non-equilibrium procedure is that the cells are first exposed to the IgG. The unbound IgG as well as the contaminting PEG is removed by rinsing prior to the addition of the labeled TSH which is then able to bind the residual unoccupied TSH receptors. The intra-assay coefficient of variation was 2.2% at a TBI value of 0.15 and 3.9% at a TBI value of 0.55 (n = 7). The inter-assay variation with a serum providing TBI activity of 0.55 was 5.5% (n = 7).

The recombinant TSH receptor assay discriminated very well between sera from normal individuals (TBI index of 0.98 ± 0.04 S.D., range 0.92 - 1.08, n = 35) and patients with untreated Graves' disease (0.49 ± 0.23 S.D., range 0.06 - 0.98, n = 93). Only four of the sera from the untreated Graves' patients were TBI negative (≥ 0.92), providing a sensitivity of 96%. In sera from patients with Graves' disease on anti-thyroid drug therapy (2.9 - 6.3 months; median of 4.3 months) for a minimum of 6 months only 73-75 were TBI positive. However the TBI indices in these patients on treatment were not as strongly positive (0.63 ± 0.18 S.D., range 0.19 - 0.97, n = 75) as in the treated group. In this group of treated patients, 2/75 were TBI negative. In 30 patients who relapsed after the withdrawal of anti-thyroid medication 29 of 30 were TBII positive. Four of 12 patients with Hashimoto's thyroiditis (33%) were positive for TBI activity. All 18 patients with non-autoimmune thyroid diseases (4 with toxic nodular goiter, 8 with single toxic adenomata, 3 with subacute thyroiditis and 3 with thyroid cancer) were TBI negative.

Comparing the data obtained with the CHO cell recombinant TSH receptor (TRAK) assay with the solubilized pig thyroid membrane assay revealed greater sensitivity with the former. Thus, of 45 sera from patients with untreated Graves' disease tested in both assays, 39/45 (86.7%) were positive in the TRAK assay and 43/45 (95.6%) were positive in the recombinant TSH receptor assay. In patients on anti-thyroid drug therapy tested with both assays, 33/47 (70.2%) and 46/47 (97.9%) were positive in the TRAK and recombinant TSH receptor assay, respectively. When measured at the time of relapse after

discontinuation of anti-thryoid drug therapy, 21/30 (70%) were positive in the TRAK assay and 29/30 (96.7%) were positive in the recombinant TSH receptor assay.

Correlation of TBI indices with thyroid stimulating immunoglobulin (TSI) bioactivity in 40 patients with TSI-positive untreated Graves' disease revealed a generally positive association between these two parameters ($R = 0.31$, $p<0.05$) (Fig. 23), however, there were many discrepancies among individual sera. TSI bioactivity was undetectable in all 4 patients with Hashimoto's thyroiditis who were TBI positive. The present data therefore indicate that the recombinant TSH receptor in CHO cells is a suitable system for detecting TBI activity in the sera of patients with autoimmune thyroid disease.

DISCUSSION

Following the demonstration that IgG in the serum of patients with Graves' disease can compete with TSH for binding to the TSH receptor, this system has been used effectively as a clinical test to detect the presence of these antibodies (Rees Smith et al., Endocr Rev. 9:106-121 (1988); Rees Smith et al., The Lancet 427-431 (1974)). The most commonly used method for detection of anti-TSH receptor antibodies (Shewring et al., Clin Endocrinol. 17:409-417 (1982)), marketed commercially as the TRAK assay, utilizes a solubilized porcine thyroid membrane as a source of TSH receptors.

While this assay is very conveniently performed and is generally sensitive and reliable, an abundant and uniformly constant source of human TSH receptor

would be an advantage. A single cell line expressing the human TSH receptor would avoid potential variability among pig thyroid preparations. Although the pig and human TSH receptors appear to be antigenically very similar, it is possible that some anti-TSH receptor antibodies would interact with higher affinity, and may thereby reduce the number of false positives. Indeed, the present data suggest that the recombinant human TSH receptor is, indeed more sensitive than the pig receptor assay, being 96% positive in untreated Graves's disease vs. 87% positive with the same sera in the TRAK assay.

Besides species specificity, there are a number of possible or probable reasons for the greater sensitivity of the recombinant TSH receptor assay relative to the solubilized pig thyroid membrane assay. First, the stably transfected CHO cells express approximately 10^5 TSH receptors per cell, 10 times as many receptors as are present on human thyroid cells (Rees Smith et al., Endocr Rev. 9:106-121 (1988)) or FRTL5 rat thyroid cells (Tramontano et al., Endocrinology 118:1945-1951 (1986)), although the number of TSH receptors on pig thyroid cells is unknown. A second probable reason for the greater sensitivity of the recombinant TSH receptor assay is the use of polyethylene glycol-precipitated serum rather than whole serum used in the solublized pig thyroid receptor assay. A comparison between these two assays was performed following exactly the protocol of the manufacturers of the TRAK assay. In our experience (data not shown), the sensitivity of the TRAK assay improves if it is modified to use a partially purified IgG preparation rather than whole serum. The purpose of using whole

serum is to greatly simplify the TRAK assay and to make it more widely available to many physicians taking care of patients with autoimmune thyroid disease.

Irrespective of the value of the different assays, the present data reinforce the concept that a TSH binding inhibition (TBI) assay does not measure the same activity as a thyroid stimulating immunoglobulin (TSI) bioassay. Thus, while a generally positive correlation exists between the TBI and TSI assays when assaying a group of sera, there are numerous sera that display a disparity between these two activities. This information is well-recognized in that anti-TSH receptor antibodies appear to be heterogeneous (Rees Smith et al., Endocr Rev. 9:106-121 (1988)). Some antibodies are predominantly TSH binding inhibitory.

Physicians utilizing the more readily available TBI assays are obtaining data that, while helpful, does not necessarily reflect the presence of thyroid stimulatory antibodies. Indeed, while all patients in our small series of patients with Hashimoto's thyroiditis were TSI negative, one third were TBI positive in a sensitive TBI assay. Measurement of TBI activity allows for the detection of autoantibodies against the TSH receptor in neonatal hypothyroidism or in following the course of unusual patients who are hypothyroid as the result of TSH receptor blocking antibodies (Rees Smith et al., Endocrin. Rev. 9:106-121 (1988)).

EXAMPLE IX

The receptors for the glycoprotein hormones, thyrotropin (TSH)[2], lutropin (LH), chorionic gonadotropin (CG) and follitropin (FSH) (Parmentier, M., et al., Science 246:1620-1622 (1989); Loosfelt, H., et al., Science 245:525-528 (1989); Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989); Libert, F., et al., Biochem. Biophys. Res. Comm. 165:1250-1255 (1989); Misrahi, M., et al., Biochem. Biophys. Res. Comm. 166:394-403 (1990); Sprengel, R., et al., Mol. Endocrinol. 4:525-530 (1990); Parmentier, M., et al., Science 246:1620-1622 (1989)) are members of both the guanine nucleotide regulatory protein-coupled receptor family and the leucine-rich glycoprotein family. There is, therefore, considerable homology in the primary structures of the extracellular (30-50%) and transmembrane regions (70-80%) of these receptors. The ligands for these receptors also belong to a heterodimeric glycoprotein hormone family that has a common α-subunit and a variable β-subunit (Ryan, R.J., et al., FASEB J. 2:2661-2669 (1988)). Homologous substitutions in the extracellular domain of the human TSH receptor with corresponding segments of the rat LH/CG receptor have demonstrated a high degree of tolerance for the retention of high affinity TSH binding, suggesting that the TSH binding region is likely to span the entire extracellular domain of the receptor, with multiple discontinuous contact sites (Example V). The chimeric receptors used in the foregoing studies contained predominantly the TSH receptor in which was substituted relatively small regions of the LH/CG receptor. Based on these data,

and in order to delineate in greater detail the contact sites between TSH and its receptor, a new series of chimeric receptors was constructed using the reverse approach, namely the substitution of relatively small regions of the TSH receptor in the extracellular domain of the LH/CG receptor. The present example reveals the importance for TSH binding of the mid-region of the TSH receptor extracellular domain (amino acid residues 171-260).

METHODS

Chimeric TSH-LH/CG receptor cDNA construction and expression. Previous human TSH-rat LH/CG receptor extracellular domain chimeras (Example V) were used to construct the new chimeric receptors, all in the eukaryotic expression vector pSV2-NEO-ECE (Nagayama, Y., et al., Biochem. Biophys. Res. Comm. 165:1184-1190 (1989)). TSH-LHR-12 was constructed by isolating the 610 bp fragment coding for domains AB from chimera TSH-LHR-2 with Sal I and Mlu I and substituting this fragment for the corresponding region in the same restriction sites in TSH-LHR-7 (Figure 24). TSH-LHR-13 was constructed by ligating the 510 bp by Sal I-Mlu I fragment of TSH-LHR-1 into the corresponding sites in TSH-LHR-7. TSH-LHR-14 was constructed by substituting the 780 bp Sal I-Bfr I (isoschizomer of Afl II) fragment of TSH-LHR-8 (coding for domains ABC) into the corresponding sites of TSH-LHR-6. TSH-LHR-15 was constructed by substituting the 780 bp Sal I-Bfr I fragment of TSH-LHR-9 for the corresponding fragment in TSH-LHR 5. Finally, TSH-LHR-16 was constructed by substituting the 780 bp Sal I-Bfr I fragment of TSH-LHR-9 for the corresponding tract in TSH-LHR-4. These

chimeric TSH-LH/CG receptor cDNAs in the expression vector pSV2-NEO-ECE were stably transfected into Chinese hamster ovary (CHO) cells by the calcium-phosphate method (Chen, C., et al., Mol. Cell. Biol. 7:2745-2752 (1987)). Surviving colonies were selected with G418 (400 μg/mL, Geneticin, GIBCO, Grand Island, NY) and pooled for study. pSV2-NEO was also transfected as a negative control.

Ligand-binding and intracellular cAMP measurements. TSH and hCG binding studies were performed as described previously (Example V; Chazenbalk, G.D., et al., Endocrinology 127:1240-1244 (1990)). Measurements of the intracellular cAMP response to hormone stimulation were also as previously described (Hirayu, H., et al., Molec. Cell. Endocrinol. 42:21-27 (1985)) with the exception that recombinant human TSH was used.

RESULTS

Of the five chimeric receptors, only TSH-LHR-14, which contains domain C of the extracellular component of the TSH receptor and domains ABDE of the LH/CG receptor, exhibited TSH binding of high affinity (Kd = 4.3×10^{-9} M). This affinity is approximately 1 order of magnitude lower than that of the wild-type TSH receptor (Kd = 3.4×10^{-10} M) (Table 3 and Figure 25A). Only low affinity binding unrelated to the TSH receptor (Kd; $\sim 10^{-7}$ M) was seen with all the other new chimeric constructs. The low affinity TSH binding to chimeras TSH-LHR-12, -13, -15 and -16 could obscure possible low affinity TSH binding to a chimeric receptor. Consistent with high affinity TSH binding, only chimera TSH-LHR-14 demonstrated a functional

response to TSH stimulation in terms of intracellular cAMP generation (EC50 for TSH of 2.5 x 10^{-7} M, approximately 70-fold higher than the EC50 of 3.4 x 10^{-9} M for the wild-type TSH receptor) (Table 3; Figure 25B).

Chimeric Receptor	Hormone Binding				cAMP Response			
	TSG		hCG		TSH		hCG	
	Kd(nM)	Kd-M / KD-Wt	Kd(nM)	Kd-M / Kd-Wt*	EC50(nM)	EC50-M / EC50-Wt	EC50(nM)	EC50-M / EC50-Wt#
Wild-type	0.34	1	und	und	3.4	1	und	und
TSH-LHR-11	und	und	0.04	1	und	und	0.3	1
TSH-LHR-12	und	und	0.04	1	und	und	0.3	1
TSH-LHR-13	und	und	und	und	und	und	und	und
TSH-LHR-14	4.3	13	0.46	12	250	74	14	47
TSH-LHR-15	und	und	und	und	und	und	und	und
TSH-LHR-16	und	und	0.07	1.8	und	und	0.3	1

Table 4: Hormone binding and function in CHO cells stably expressing the TSH-LH/CG receptor chimeras depicted in Figure 1.

M, mutant; Wt, wild type; und, undetectable; *, relative to the Kd for hCG binding to chimera TSH-LHR-11; #, relative to the EC50 for hCG stimulation of chimera TSH-LHR-11.

Each value represents the mean of data obtained with two pools of clones from two separate transfections, each transfection measured in duplicate.

Examination of the chimeric TSH/LH/CG receptors for hCG binding and functional responsiveness to hCG stimulation revealed that the affinity of chimera TSH-LHR-14 was approximately 1 order of magnitude lower than that of chimera TSH-LHR-11, in which the entire LH/CG extracellular domain replaces the corresponding region in the TSH receptor (Table 4, Figure 26A). Consistent with this lower affinity, chimera TSH-LHR-

14 had a blunted cAMP response to hCG stimulation (EC50 approximately 50-fold higher than that of chimera TSH-LHR-11 (Table 4; Figure 26B). Chimeras TSH-LHR-12 and TSH-LHR-16 (containing only extracellular domain A and domain E of the TSH receptor, respectively) retained high affinity hCG binding (Kd = 4-6 x 10^{-11} M) comparable to that of chimera TSH-LHR-11. Consistent with this high affinity binding, the EC50 for hCG stimulation of cAMP generation in these two chimeric receptors was similar to that for TSH-LHR-11 (Table 4; Figure 26B). Chimeras TSH-LHR-13 and TSH-LHR-15 exhibited neither hCG binding nor functional responsiveness to hCG stimulation.

DISCUSSION

The present study demonstrates that only domain C (amino acid residues 171-260) in the mid-region of the extracellular component of the TSH receptor can, by itself, confer high affinity TSH binding, with an affinity approximately one order of magnitude lower than that of the wild-type receptor. Domain C in the TSH receptor has previously been shown to be important in signal transduction (Example V). Taken together, these data suggest that extracellular domain C in the TSH receptor plays a vital role in receptor function. Similarly, the substitution of TSH receptor extracellular domain C (but not domains A or E) for the corresponding region in the extracellular component of the LH/CG receptor decreases the affinity for hCG binding, thereby indicating that domain C is also important in hCG binding to its receptor. The homology between the human TSH and rat LH/CG receptors

in domains A through E is 26% (the putative signal peptide is excluded), 43%, 51%, 19% and 39%, respectively. Surprisingly, domain C is the most conserved of the five domains. Sequence divergence may therefore not be a good predictor for defining regions responsible for functional differences in the glycoprotein hormone receptor family.

Chimeras TSH-LHR-13 and TSH-LHR-15, in which domains B and D of the TSH receptor are substituted into the extracellular region of the LH/CG receptor, respectively, respond to neither TSH nor hCG.

In previous studies (Example V), chimeric receptors containing only TSH receptor extracellular domains ABC or CD bound TSH with high affinity. Thus domain C, together with domains AB or with D, can fully restore TSH binding to the high affinity of wild-type receptor. However, domains DE, lacking domain C, also display high affinity TSH binding comparable to the wild-type receptor, although this chimera cannot transduce a signal. Taken together, all these data suggest that there are at least three distinct, partially overlapping sites that can confer high affinity TSH binding.

Recent studies suggest that both α- and β-subunits of the glycoprotein hormones are involved in receptor-binding (Ryan, R.J., et al., FASEB J. 2:2661-2669 (1988)), presumably binding to distinct sites on the extracellular domain of the receptor. The β-subunit, which varies among the different glycoprotein hormones and not the common α-subunit, confers hormone binding specificity. The chimeric receptor approach is therefore not a good means for identifying regions of α-subunit binding site(s) which may be conserved in all members of the receptor family. For example, non-

homologous substitutions in a region of the TSH receptor have been shown to affect TSH binding (Wadsworth, H.L., et al., Science 249:1423-1425 (1990)). A corollary of these chimeric homologous substitution data is that the alterations in ligand binding observed in the previous (Example V) and present studies are likely to reflect β-subunit binding.

Besides TSH binding to the TSH receptor, the data also provide some information with respect to hCG binding to the LH/CG receptor. As for TSH binding, the binding of hCG to its receptor is complex and is likely to involve multiple contact sites in the extracellular domain of the receptor, and may be summarized as follows: a) The binding of hCG to its receptor appears to be less tolerant of homologous substitution than is the binding of TSH to the TSH receptor (Example V); b) Domains A and E of the hCG receptor may be individually substituted without affecting high affinity hCG binding (present study), play a role in hCG binding to its receptor; and, c) the addition of the D domain (TSH-LHR-16) to the ABC domains (TSH-LHR-9) (Example V) fully restores high affinity hCG binding, suggesting that domain D also plays a role in hCG binding.

EXAMPLE X

Due to the complexity of the results obtained in Examples VIII and IX, a series of experiments designed to demonstrate the TSH receptor domains involved in TSH binding, TSH stimulation, TSI activity, and TBII activity was performed (Table 5).

TSH receptor/LH receptor chimeras were used to assess the Thyroid Stimulating Immunoglobulin (TSI) activity of the various domains of the TSH receptor. Purified IgG from 11 different Graves' disease sera was tested, as well as 4 Hashimoto's thyroiditis sera not containing TSI activity. The chimeras used are described in Examples VIII and IX.

The results demonstrate that only TSI stimulates the wild type TSH receptor (as expected) and the chimera containing the ABC domain. Data are presented in Figure 27.

The data show that TSI stimulate only the wild type TSH receptor and the chimera containing the ABC domain.

TSH binding inhibitory immunoglobulin (TBII) activity was assessed using selected TSH receptor/LH receptor chimeras. The data indicate that TBII activity is much greater using the chimera containing the DE domain of the TSH receptor than when using the chimera containing the ABC domain of the receptor. Data are presented in Figure 28. The data show that TBII activity is much greater with the chimera containing the DE domain of the TSH receptor than the chimera containing the ABC domain of the receptor.

The ability of Hashimoto's sera containing pure TSH binding inhibitory activity to inhibit TSI activity was also assessed. The wild type TSH receptor and chimera 6 containing the ABC domains were used in the assay. The data indicate that TBII only blocks TSI activity in the wild-type TSH receptor, and not in the ABC domains of the TSH receptor. (Table 5).

		WT-TSHR	TSH-LHR-6
Graves' IgG4	+pooled normal IgG	219.0+15.3	182.0+18.3
	+Hashimoto's IgG12	104.0+18.7*	195.6+20.4
	+Hashimoto's IgG13	137.5+ 8.3*	213.0+34.1
Graves' IgG5	+pooled normal IgG	228.4+20.6	247.8+22.3
	+Hashimoto's IgG12	142.4+15.7*	247.8+14.9
	+Hashimoto's IgG13	124.0+10.5*	239.2+28.0

Table 5: Ability of Hashimoto's sera containing pure TSH binding inhibitory activity to inhibit Thyroid Stimulating Immunoglobulin activity. Purified IgG from the indicated sera were used. The numbers ± SD (n=3) represent cellular cAMP levels, normalized to 100%, i.e. 100 = no stimulation. WT-TSHR - wild type TSH receptor; TSH-LHR-6 contains only the ABC domains of the extracellular domain of the TSH receptor.

The data indicate that TBII blocks TSI activity in the wild-type TSH receptor, and not in the ABC domains of the TSH receptor.

	WT-TSHR	TSH-LHR-6	TSH-LHR-10	TSH-LHR-9
TSHR domains	ABCDE	ABC	CD	DE
TSH binding	+	+	+	+
TSH stimulation	+	+	+	-
TSI activity	+	+	-	-
TBII activity	+	-/+	-	+
TSI activity inhibition by blocking Ab	+	-	ND	ND

Table 6: Summary of data shown in Figures 27, 28 and Table 5.

CLAIMS

What is claimed is:

1. Recombinant, enzymatically active thyrotropin receptor.

2. A DNA sequence comprising the DNA sequence of Figure 1.

3. A amino acid sequence comprising the nucleotide sequence of Figure 1.

4. A plasmid comprising the DNA sequence of claim 2.

5. A host cell transformed by the plasmid of claim 4.

6. A method of producing thyrotropin receptor, comprising culturing the transformed cell of claim 5, under conditions allowing expression of thyrotropin receptor, and recovering said thyrotropin receptor.

7. An antibody against the recombinant thyrotropin receptor of claim 1.

8. The antibody of claim 7, wherein said antibody is selected from the group consisting of a monoclonal antibody, polyclonal antibody, an anti-idiotypic antibody, or an anti anti-idiotypic antibody.

9. Recombinant enzymatically active thyrotropin receptor wherein a 50 amino acid region comprising residues 317-366 has been deleted.

10. An antibody against the recombinant thyrotropin receptor of claim 9.

11. The antibody of claim 10, wherein, said antibody is selected from a group consisting of a monoclonal antibody, polyclonal antibody, an anti-idiotypic antibody, or an anti anti-idiotypic antibody.

12. A recombinant thyrotropin receptor peptide comprised of the eight amino acid region comprising residues 38-45 of the thyrotropin receptor of claim 1.

13. An antibody against the recombinant thyrotropin receptor peptide of claim 12.

14. The antibody of claim 13 wherein, said antibody is selected from a group consisting of a monoclonal antibody, polyclonal antibody, an anti-idiotypic antibody, or an anti anti-idiotypic antibody.

15. Recombinant thyrotropin receptor wherein an eight amino acid region comprising residues 38-45 have been deleted or replaced.

16. A recombinant thyrotropin receptor peptide comprised of the mid-region domain C comprising amino acid residues 171-260 of the thyrotropin receptor of claim 1.

17. An antibody against the recombinant thyrotropin receptor peptide of claim 16.

18. The antibody of claim 17, wherein said antibody is selected from a group consisting of a monoclonal antibody, polyclonal antibody, an antiidiotypic antibody, or an anti anti-idiotypic antibody.

19. Recombinant thyrotropin receptor wherein the mid-region domain C comprising amino acid residues 171-260 has been deleted or replaced.

20. A method of detecting thyrotropin receptor in a sample comprising contacting said sample with the antibody of any of claims 7 or 8, wherein said antibody is detectably labeled, so as to form a complex between said thyrotropin receptor in said sample and said detectably labeled antibody, and detecting the complexed or uncomplexed detectably labeled antibody.

21. A kit for the detection of thyrotropin receptor in a sample, comprising container means comprising one or more containers, wherein one of said containers comprises detectably labeled antibody against thyrotropin receptor.

22. A method of detecting an antibody to thyrotropin receptor in a sample comprising contacting said sample with the thyrotropin receptor of claim 1, wherein said thyrotropin receptor is detectably labeled, so as to form a complex between the antibody to said thyrotropin receptor in said sample and said

detectably labeled thyrotropin receptor, and detecting the complexed or uncomplexed detectably labeled thyrotropin receptor.

23. A method for detecting an antibody to thyrotropin in a sample, comprising contacting said sample with Chinese Hamster ovary (CHO) cells stably transfected with human thyrotropin receptor cDNA wherein said CHO cells are expressing the functional thyrotropin receptor of claim 1, wherein said thyrotropin receptor is detectably labeled so as to form a complex between the antibody to said thyrotropin receptor in said sample and said detectably labeled thyrotropin receptor, and detecting the complexed or uncomplexed detectably labeled thyrotropin receptor.

24. A method of differentiating between autoimmune and non-autoimmune forms of thyrotoxicosis comprising contacting a sample with the antibody of any of claims 7 or 8.

25. The method of claim 24, wherein said antibody is detectably labeled, so as to form a complex between said thyrotropin receptor in said sample and said detectably labeled antibody and detecting the complexed or uncomplexed detectably labeled antibody.

26. A kit for the detection of an antibody to thyrotropin receptor in a sample, comprising container means comprising one or more containers, wherein one of said containers comprises detectably labeled thyrotropin receptor.

27. A pharmaceutical preparation comprising the recombinant thyrotropin receptor of claim 1.

28. A method of treating thyrotoxicosis with the pharmaceutical preparation of claim 13.

29. A T cell which is specific for the autoimmune T-cell receptor for thyrotropin receptor.

30. A peptide which is specific for the autoimmune T-cell receptor for thyrotropin receptor.

31. A suppressor T cell specific for anti-thyrotropin receptor auto-antibody.

32. A pharmaceutical preparation comprising the T cell, peptide, or suppressor T cell of claims 29-31.

33. A method of treating thyrotoxicosis with the pharmaceutical preparation of claim 32.

34. A pharmaceutical preparation comprising the recombinant thyrotropin receptor peptide of claim 12.

35. A method of treating thyrotoxicosis with the pharmaceutical preparation of claim 34.

36. A pharmaceutical preparation comprising the recombinant thyrotropin receptor peptide of claim 16.

37. A method of treating thryotoxicisis with the pharmaceutical preparation of claim 36.

```
   1: CCGCTCCCGGGTCTCCTTTGGCCTGGGGTAACCCGAGGTGCAGAGCTGAGAATGAGGCGATTTCGGAGGATGGAGAAATAGCCCCGAGTC

  91: CCGTGGAAAATGAGGCCGGCGGACTTGCTGCAGCTGGTGCTGCTGCTCGACCTGCCCAGGGACCTGGGCGGAATGGGGTGTTCGTCTCCA
   1:           M  R  P  A  D  L  L  Q  L  V  L  L  L  D  L  P  R  D  L  G  G  M  G  C  S  S  P

 181: CCCTGCGAGTGCCATCAGGAGGAGGACTTCAGAGTCACCTGCAAGGATATTCAACGCATCCCCAGCTTACCGCCCAGTACGCAGACTCTG
  28:  P  C  E  C  H  Q  E  E  D  F  R  V  T  C  K  D  I  Q  R  I  P  S  L  P  P  S  T  Q  T  L

 271: AAGCTTATTGAGACTCACCTGAGAACTATTCCAAGTCATGCATTTTCTAATCTGCCCAATATTTCCAGAATCTACGTATCTATAGATGTG
  58:  K  L  I  E  T  H  L  R  T  I  P  S  H  A  F  S  N  L  P  N  I  S  R  I  Y  V  S  I  D  V

 361: ACTCTGCAGCAGCTGGAATCACACTCCTTCTACAATTTGAGTAAAGTGACTCACATAGAAATTCGGAATACCAGGAACTTAACTTACATA
  88:  T  L  Q  Q  L  E  S  H  S  F  Y  N  L  S  K  V  T  H  I  E  I  R  N  T  R  N  L  T  Y  I

 451: GACCCTGATGCCCTCAAAGAGCTCCCCCTCCTAAAGTTCCTTGGCATTTTCAACACTGGACTTAAAATGTTCCCTGACCTGACCAAAGTT
 118:  D  P  D  A  L  K  E  L  P  L  L  K  F  L  G  I  F  N  T  G  L  K  M  F  P  D  L  T  K  V

 541: TATTCCACTGATATATTCTTTATACTTGAAATTACAGACAACCCTTACATGACGTCAATCCCTGTGAATGCTTTTCAGGGACTATGCAAT
 148:  Y  S  T  D  I  F  F  I  L  E  I  T  D  N  P  Y  M  T  S  I  P  V  N  A  F  Q  G  L  C  N

 631: GAAACCTTGACACTGAAGCTGTACAACAATGGCTTTACTTCAGTCCAAGGATATGCTTTCAATGGGACAAAGCTGGATGCTGTTTACCTA
 178:  E  T  L  T  L  K  L  Y  N  N  G  F  T  S  V  Q  G  Y  A  F  N  G  T  K  L  D  A  V  Y  L

 721: AACAAGAATAAATACCTGACAGTTATTGACAAAGATGCATTTGGAGGAGTATACAGTGGACCAAGCTTGCTGGACGTGTCTCAAACCAGT
 208:  N  K  N  K  Y  L  T  V  I  D  K  D  A  F  G  G  V  Y  S  G  P  S  L  L  D  V  S  Q  T  S

 811: GTCACTGCCCTTCCATCCAAAGGCCTGGAGCACCTGAAGGAACTGATAGCAAGAAACACCTGGACTCTTAAGAAACTTCCACTTTCCTTG
 238:  V  T  A  L  P  S  K  G  L  E  H  L  K  E  L  I  A  R  N  T  W  T  L  K  K  L  P  L  S  L
```

FIG. 1.1

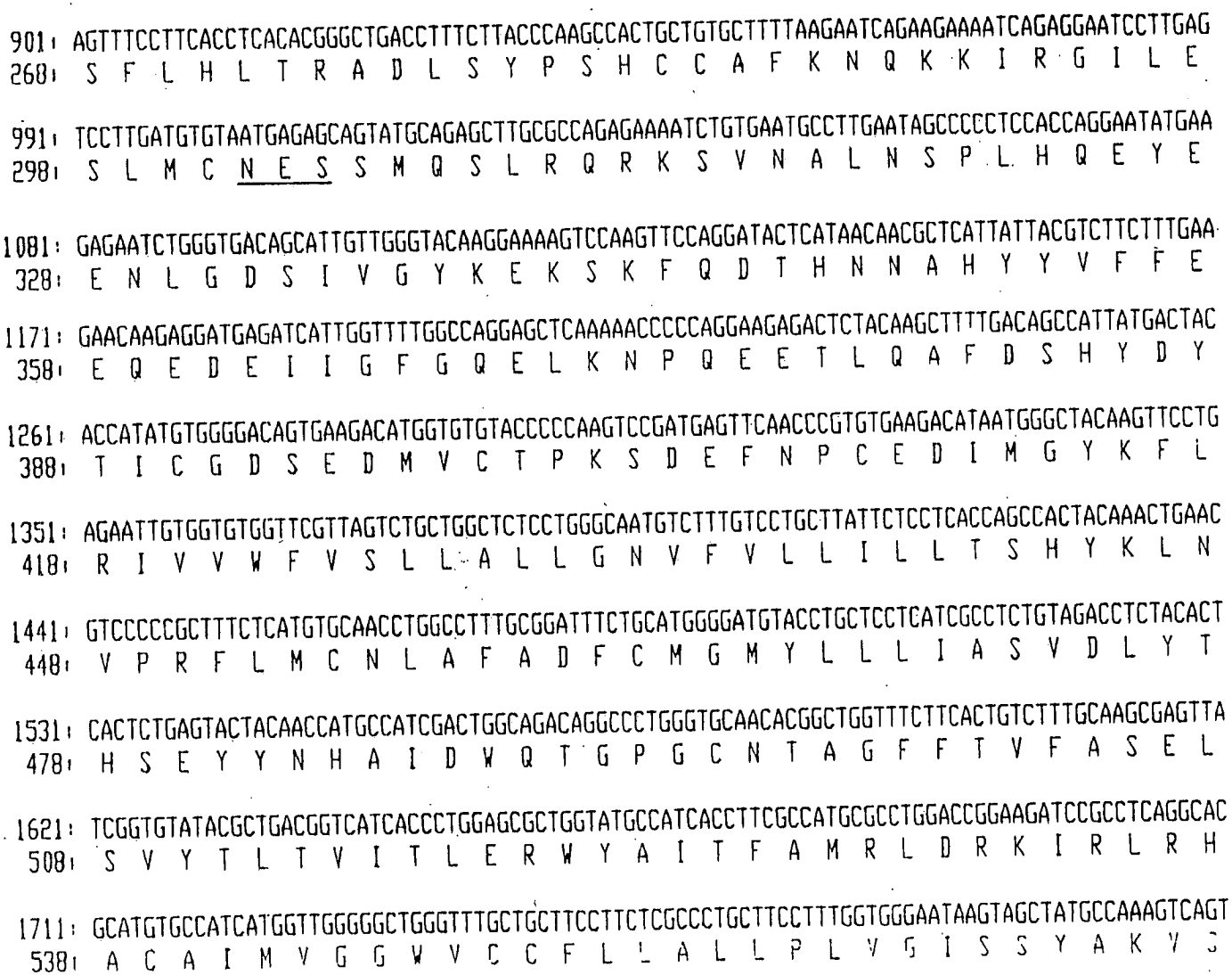

901: AGTTTCCTTCACCTCACACGGGCTGACCTTTCTTACCCAAGCCACTGCTGTGCTTTTAAGAATCAGAAGAAAATCAGAGGAATCCTTGAG
268: S F L H L T R A D L S Y P S H C C A F K N Q K K I R G I L E
991: TCCTTGATGTGTAATGAGAGCAGTATGCAGAGCTTGCGCCAGAGAAAATCTGTGAATGCCTTGAATAGCCCCCTCCACCAGGAATATGAA
298: S L M C N E S S M Q S L R Q R K S V N A L N S P L H Q E Y E
1081: GAGAATCTGGGTGACAGCATTGTTGGGTACAAGGAAAAGTCCAAGTTCCAGGATACTCATAACAACGCTCATTATTACGTCTTCTTTGAA
328: E N L G D S I V G Y K E K S K F Q D T H N N A H Y Y V F F E
1171: GAACAAGAGGATGAGATCATTGGTTTTGGCCAGGAGCTCAAAAACCCCCAGGAAGAGACTCTACAAGCTTTTGACAGCCATTATGACTAC
358: E Q E D E I I G F G Q E L K N P Q E E T L Q A F D S H Y D Y
1261: ACCATATGTGGGGACAGTGAAGACATGGTGTGTACCCCCAAGTCCGATGAGTTCAACCCGTGTGAAGACATAATGGGCTACAAGTTCCTG
388: T I C G D S E D M V C T P K S D E F N P C E D I M G Y K F L
1351: AGAATTGTGGTGTGGTTCGTTAGTCTGCTGGCTCTCCTGGGCAATGTCTTTGTCCTGCTTATTCTCCTCACCAGCCACTACAAACTGAAC
418: R I V V W F V S L L A L L G N V F V L L I L L T S H Y K L N
1441: GTCCCCCGCTTTCTCATGTGCAACCTGGCCTTTGCGGATTTCTGCATGGGGATGTACCTGCTCCTCATCGCCTCTGTAGACCTCTACACT
448: V P R F L M C N L A F A D F C M G M Y L L L I A S V D L Y T
1531: CACTCTGAGTACTACAACCATGCCATCGACTGGCAGACAGGCCCTGGGTGCAACACGGCTGGTTTCTTCACTGTCTTTGCAAGCGAGTTA
478: H S E Y Y N H A I D W Q T G P G C N T A G F F T V F A S E L
1621: TCGGTGTATACGCTGACGGTCATCACCCTGGAGCGCTGGTATGCCATCACCTTCGCCATGCGCCTGGACCGGAAGATCCGCCTCAGGCAC
508: S V Y T L T V I T L E R W Y A I T F A M R L D R K I R L R H
1711: GCATGTGCCATCATGGTTGGGGGCTGGGTTTGCTGCTTCCTTCTCGCCCTGCTTCCTTTGGTGGGAATAAGTAGCTATGCCAAAGTCAGT
538: A C A I M V G G W V C C F L L A L L P L V G I S S Y A K V S

FIG. 1.2

```
1801: ATCTGCCTGCCCATGGACACCGAGACCCCTCTTGCTCTGGCATATATTGTTTTTGTTCTGACGCTCAACATAGTTGCCTTCGTCATCGTC
 568:  I  C  L  P  M  D  T  E  T  P  L  A  L  A  Y  I  V  F  V  L  T  L  N  I  V  A  F  V  I  V

1891: TGCTGCTGTCATGTGAAGATCTACATCACAGTCCGAAATCCGCAGTACAACCCAGGGGACAAAGATACCAAAATTGCCAAGAGGATGGCT
 598:  C  C  C  H  V  K  I  Y  I  T  V  R  N  P  Q  Y  N  P  G  D  K  D  T  K  I  A  K  R  M  A

1981: GTGTTGATCTTCACCGACTTCATATGCATGGCCCCAATCTCATTCTATGCTCTGTCAGCAATTCTGAACAAGCCTCTCATCACTGTTAGC
 628:  V  L  I  F  T  D  F  I  C  M  A  P  I  S  F  Y  A  L  S  A  I  L  N  K  P  L  I  T  V  S

2071: AACTCCAAAATCTTGCTGGTACTCTTCTATCCACTTAACTCCTGTGCCAATCCATTCCTCTATGCTATTTTCACCAAGGCCTTCCAGAGG
 658:  N  S  K  I  L  L  V  L  F  Y  P  L  N  S  C  A  N  P  F  L  Y  A  I  F  T  K  A  F  Q  R

2161: GATGTGTTCATCCTACTCAGCAAGTTTGGCATCTGTAAACGCCAGGCTCAGGCATACCGGGGGCAGAGGGTTCCTCCAAAGAACAGCACT
 688:  D  V  F  I  L  L  S  K  F  G  I  C  K  R  Q  A  Q  A  Y  R  G  Q  R  V  P  P  K  N  S  T

2251: GATATTCAGGTTCAAAAGGTTACCCACGACATGAGGCAGGGTCTCCACAACATGGAAGATGTCTATGAACTGATTGAAAACTCCCATCTA
 718:  D  I  Q  V  Q  K  V  T  H  D  M  R  Q  G  L  H  N  M  E  D  V  Y  E  L  I  E  N  S  H  L

2341: ACCCCAAAGAAGCAAGGCCAAATCTCAGAAGAGTATATGCAAACGGTTTTGTAAGTTAACACTACACTACTCACAATGGTAGGGGAACTT
 748:  T  P  K  K  Q  G  Q  I  S  E  E  Y  M  Q  T  V  L

2431: ACAAAATAATAGTTTCTTGAATATGCATTCCAATCCCAT
```

FIG. 1.3

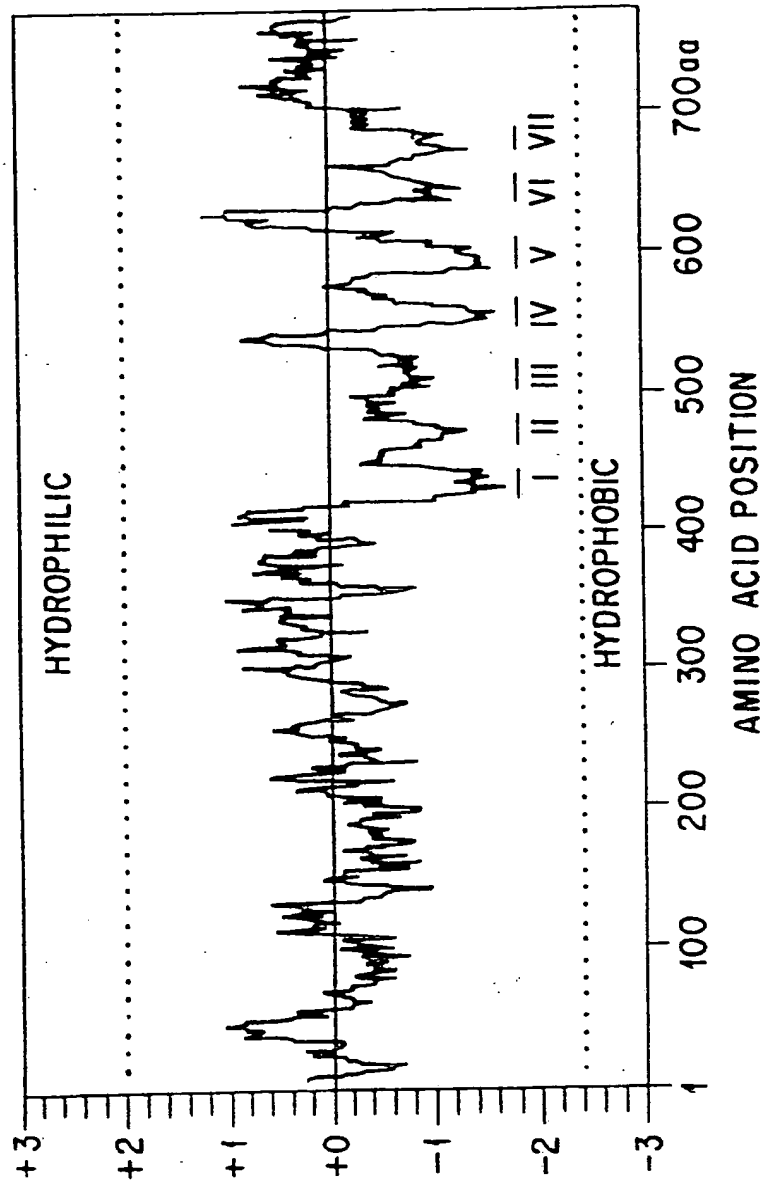

HYDROPHILIC
HYDROPHOBIC
I II III IV V VI VII
+3
+2
+1
+0
-1
-2
-3
1
100
200
300
400
500
600
700aa
AMINO ACID POSITION

FIG. 2

```
htshrec : MRPAD.....LLQLVLLLDLPRDLGGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQ
piglhrec : --RRSlalrl--A-L--PPPLPQTLLGAPCPE--S-RPDGAL.........-C-GPRAGLS

htshrec : TLKLIETHLRTIPSHAFSNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTRNLT
piglhrec : R-S-TYLPIKV---Q--RG-NEVVK-EI-QSDS-EKI-ANA-D--LNLSE-L-Q--K--V

htshrec : YIDPDALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGL
piglhrec : --E-G-FTN--R--Y-S-C---IRKL--V--IF-SEFN-----C--LHI-TV-A-----M

htshrec : CNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQ
piglhrec : N--SI-----G---EEI-SH-----T-ISLE-KE-AH-KKMHN---R-A.R---I--I-S

htshrec : TSVTALPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRGI
piglhrec : -KLQ----Y---SIQT---TSSYS-----SREK-TN-LD-T-T-------GR-LPTKEQN

htshrec : LESLMCNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYVF
piglhrec : FSFSIFKNF-K-CESTARRP-.....

htshrec : FEEQEDEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCEDIMGYK
piglhrec : ..........NET-YSAIFAESELS-WD---GF-.SPKTLQ-A-EP-A----------D

htshrec : FLRIVVWFVSLLALLGNVFVLLILLTSHYKLNVPRFLMCNLAFADFCMGMYLLLIASVDL
piglhrec : ---VLI-LINI--IM---T--FV--------T---------S-------L---------A
```

FIG. 3.1

```
htshrec : YTHSEYYNHAIDWQTGPGCNTAGFFTVFASELSVYTLTVITLERWYAITFAMRLDRKIRL
piglhrec : Q-KGQ----------N--SV----------HT--Y-IQ--Q-L--

htshrec : RHACAIMVGGWVCCFLLALLPLVGISSYAKVSICLPMDTETPLALAYIVFVLTLNIVAFV
piglhrec : ---IP--L---LFST-I-M-----V---M---------V--T-SQV--LTI-I--V---I

htshrec : IVCCCHVKIYITVRNPQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLIT
piglhrec : -I-A-YI---FA-Q--ELMATN-------K----M----T-------F-I--A-KV----

htshrec : VSNSKILLVLFYPLNSCANPFLYAIFTKAFQRDVFILLSKFGICKRQAQAYRGQRVPPKN
piglhrec : -T---V-------V---------------R--F-L----S-C--H--EL--RKDFSAYC

htshrec : STDIQVQKVTHDMRQGLHNMEDVYELIENSHLTPKKQGQISEEYMQTVL
piglhrec : KNGFTGSNKPSRSTLK-TTLQCQ-STVMDKTCYKD.............C
```

FIG. 3.2

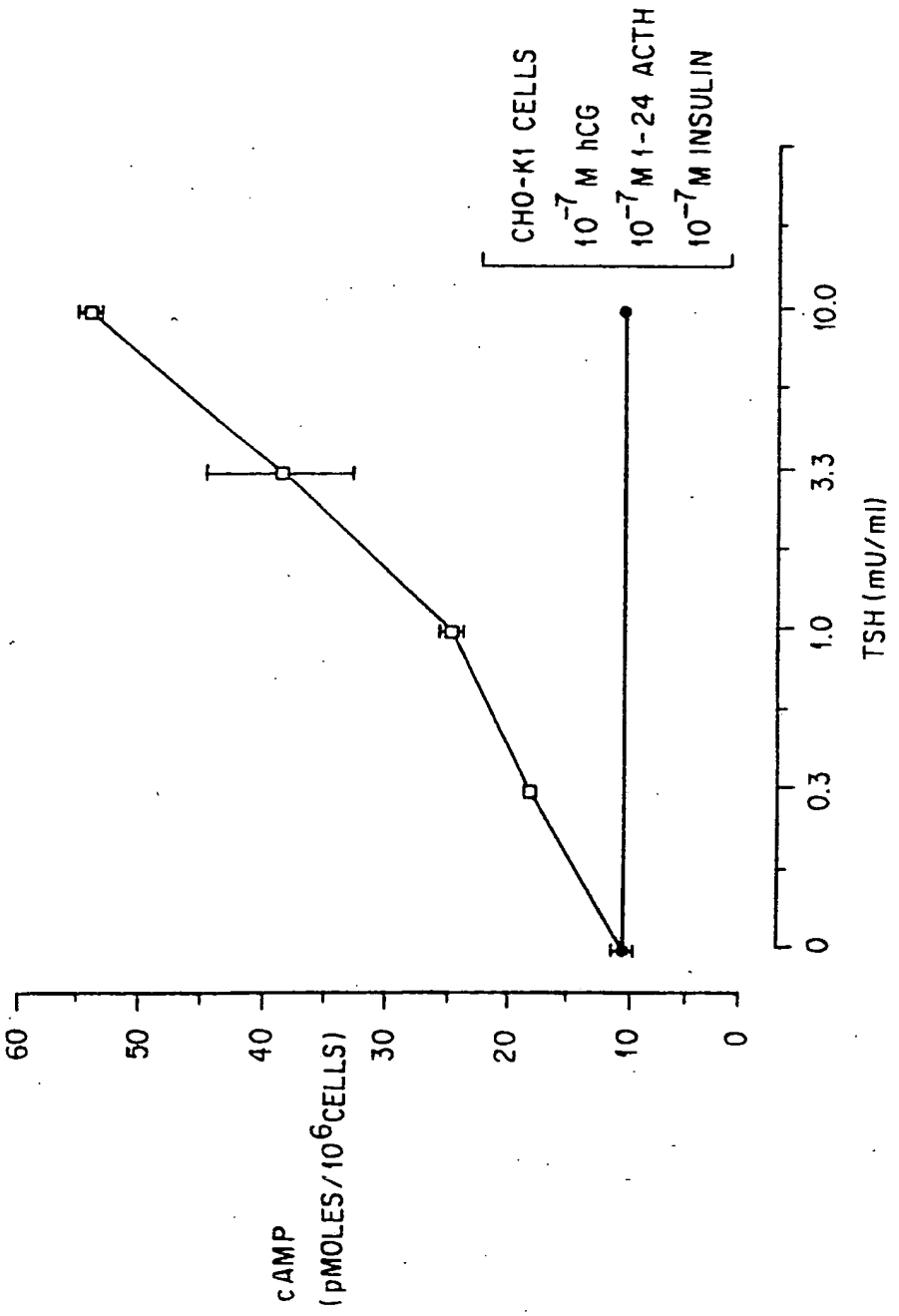

CHO-K1 CELLS
10^{-7} M hCG
10^{-7} M 1-24 ACTH
10^{-7} M INSULIN
cAMP
(pMOLES/10^6CELLS)
0
10
20
30
40
50
60
TSH (mU/ml)
0
0.3
1.0
3.3
10.0

FIG. 4

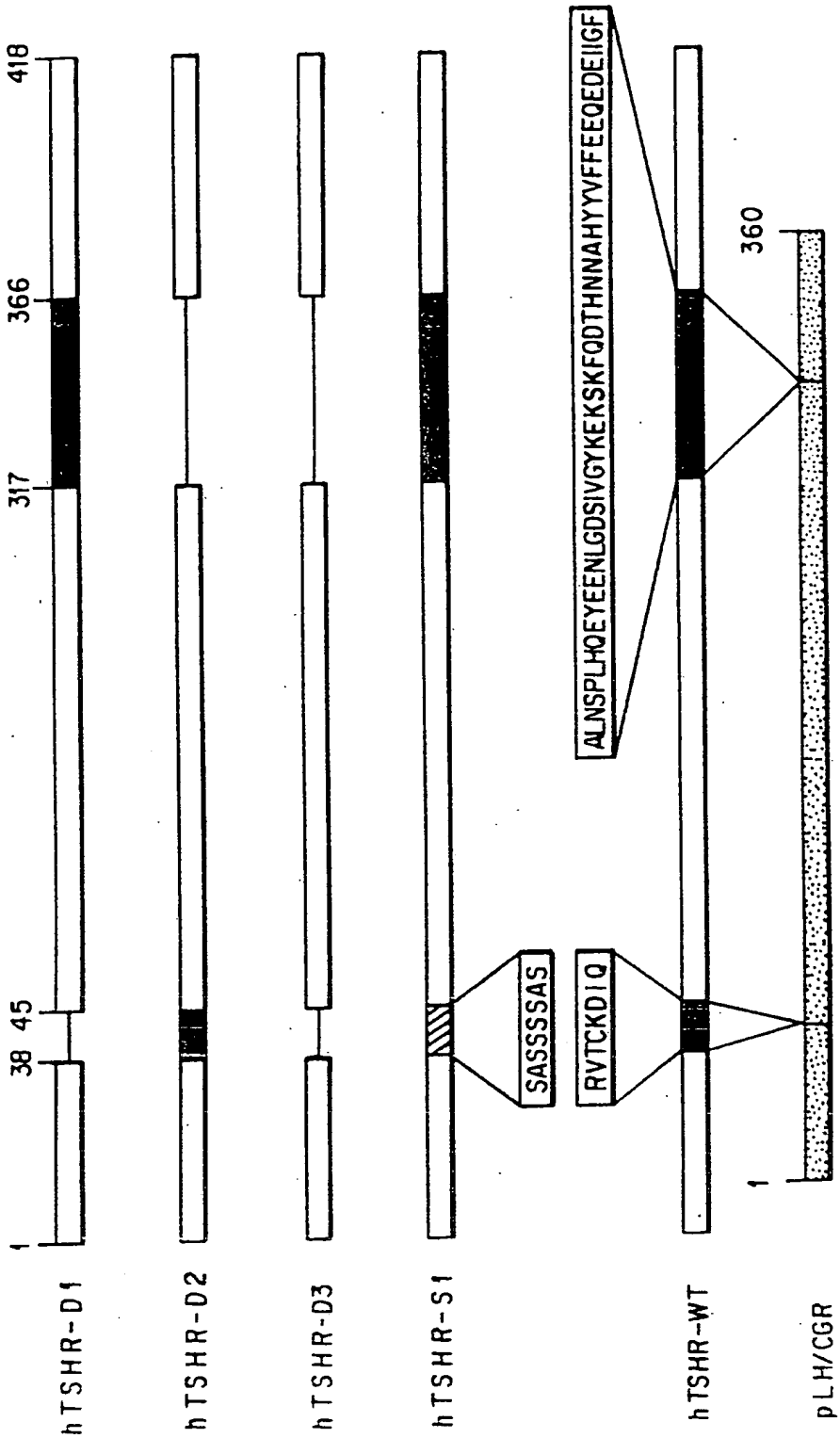

418
366
317
45
38
1
360
1
hTSHR-D1
hTSHR-D2
hTSHR-D3
hTSHR-S1
hTSHR-WT
pLH/CGR
SASSSSAS
RVTCKDIQ
ALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYVFFEEQEDEIIGF

FIG. 5

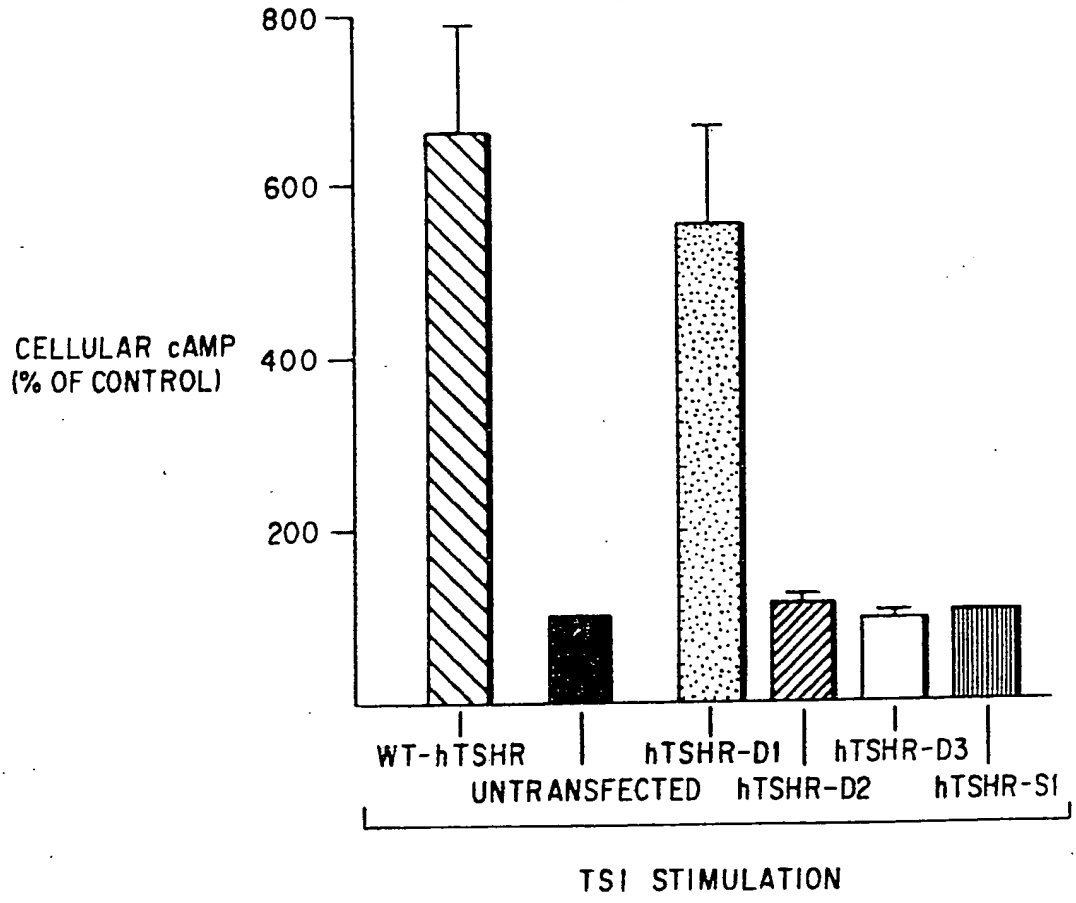

800
600
400
200
CELLULAR cAMP
(% OF CONTROL)
WT-hTSHR
UNTRANSFECTED
hTSHR-D1
hTSHR-D2
hTSHR-D3
hTSHR-S1
TSI STIMULATION

FIG. 6

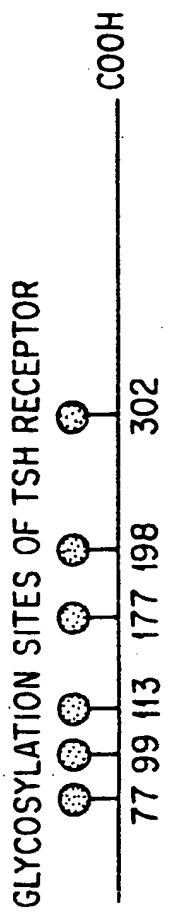


FIG. 7A

MUTANT TSH RECEPTOR cDNA CONSTRUCTS

CONSTRUCT	AMINO ACID CHANGE	DNA CODON MUTATION
WT-TSHR	NONE	
TSHR-Gln 77	Asn TO Gln	AAT TO CAG
TSHR-Gln 99	Asn TO Gln	AAT TO CAG
TSHR-Gln 113	Asn TO Gln	AAC TO CAG
TSHR-Gln 177	Asn TO Gln	AAT TO CAG
TSHR-Gln 198	Asn TO Gln	AAT TO CAG
TSHR-Gln 302	Asn TO Gln	AAT TO CAG
TSHR-Gln x6	ALL6 POTENTIAL GLYCOSYLATION SITES MUTATED	

FIG. 7B

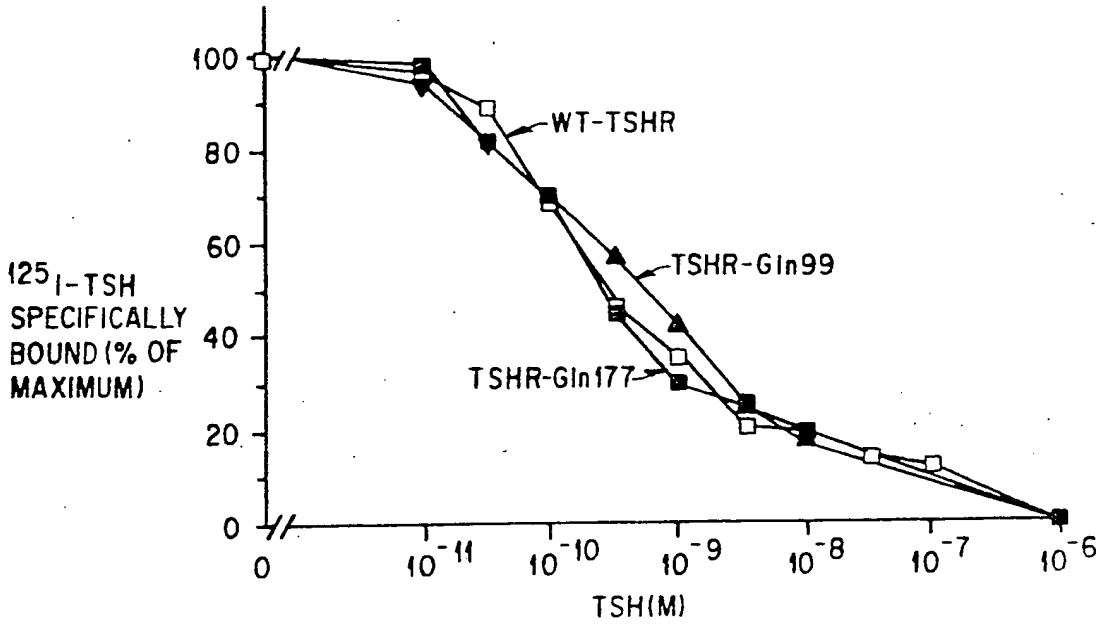

100
80
60
40
20
0
^{125}I-TSH SPECIFICALLY BOUND (% OF MAXIMUM)
WT-TSHR
TSHR-Gln99
TSHR-Gln177
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
TSH(M)

FIG. 8A

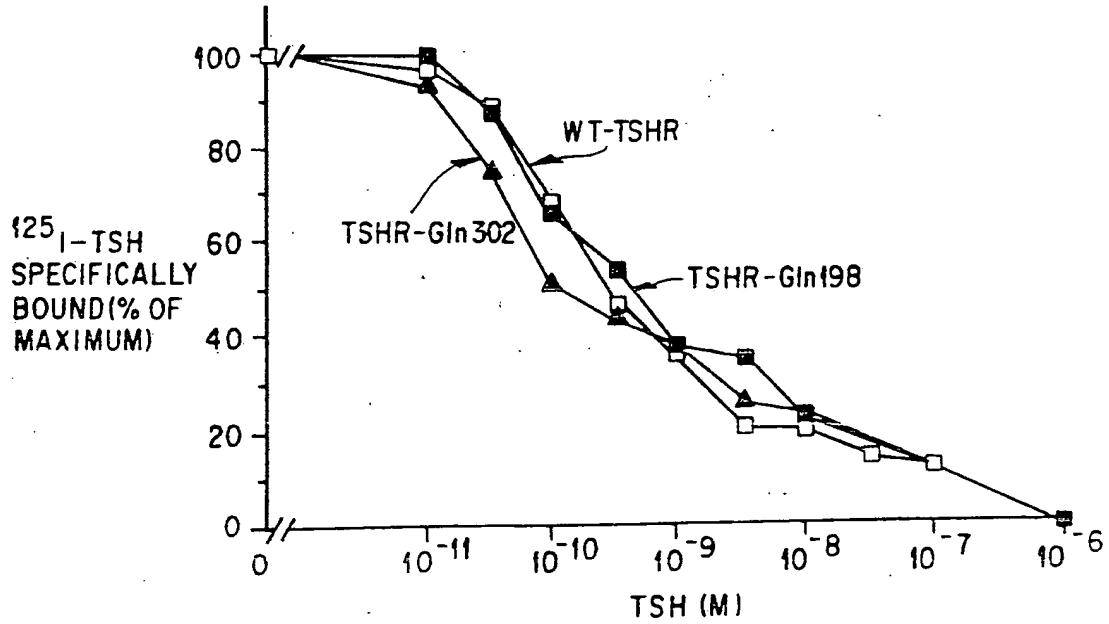

100
80
60
40
20
0
^{125}I-TSH SPECIFICALLY BOUND (% OF MAXIMUM)
WT-TSHR
TSHR-Gln302
TSHR-Gln198
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
TSH (M)

FIG. 8B

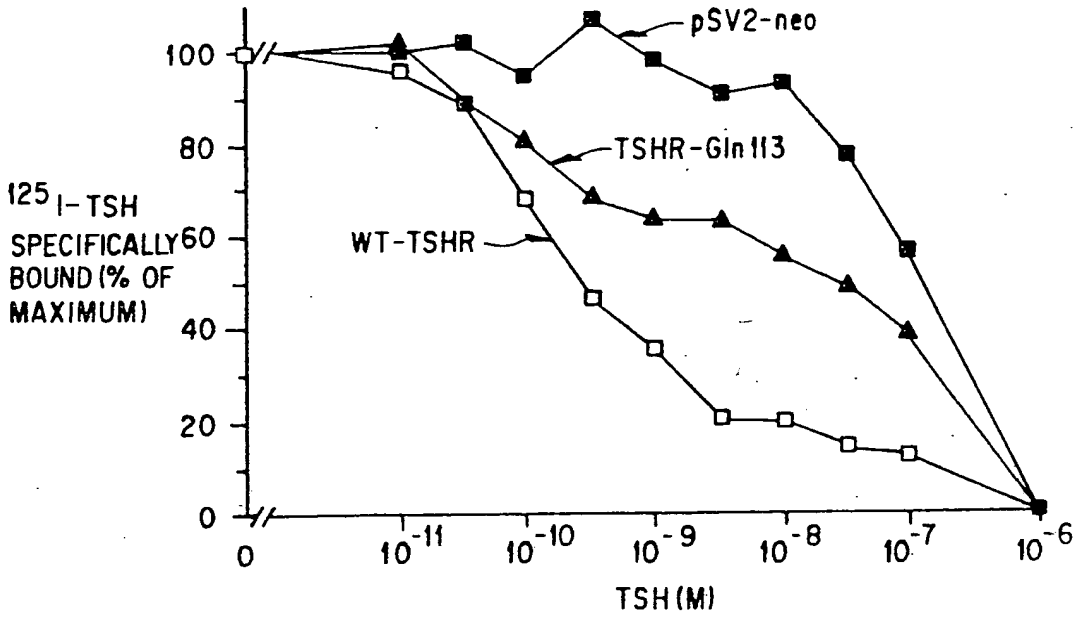

pSV2-neo
TSHR-Gln 113
WT-TSHR
^{125}I-TSH SPECIFICALLY BOUND (% OF MAXIMUM)
100
80
60
40
20
0
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
TSH (M)

FIG. 8C

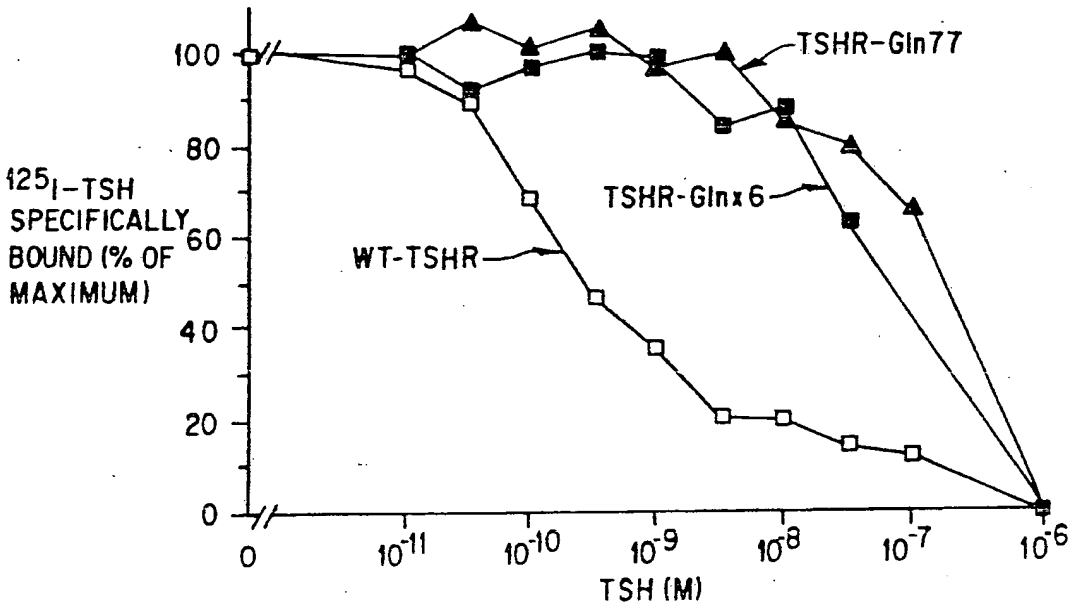

TSHR-Gln 77
TSHR-Glnx 6
WT-TSHR
^{125}I-TSH SPECIFICALLY BOUND (% OF MAXIMUM)
100
80
60
40
20
0
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
TSH (M)

FIG. 8D

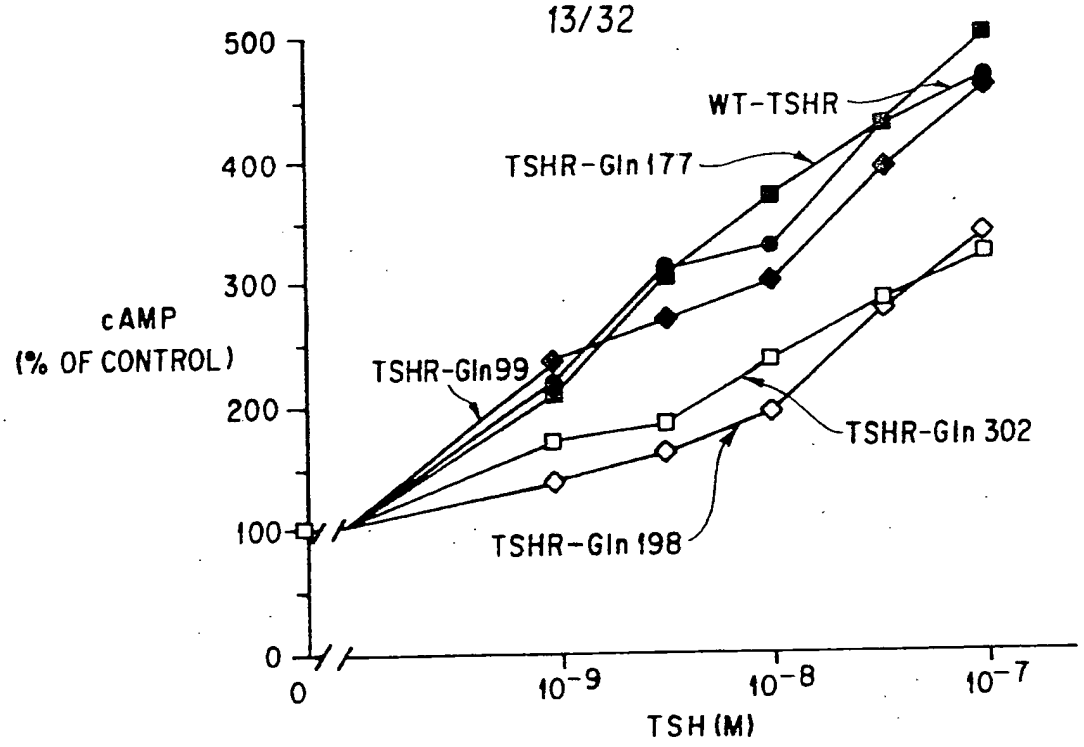

500
400
300
200
100
0
cAMP
(% OF CONTROL)
WT-TSHR
TSHR-Gln177
TSHR-Gln99
TSHR-Gln 302
TSHR-Gln 198
0
10-9
10-8
10-7
TSH (M)

FIG. 9A

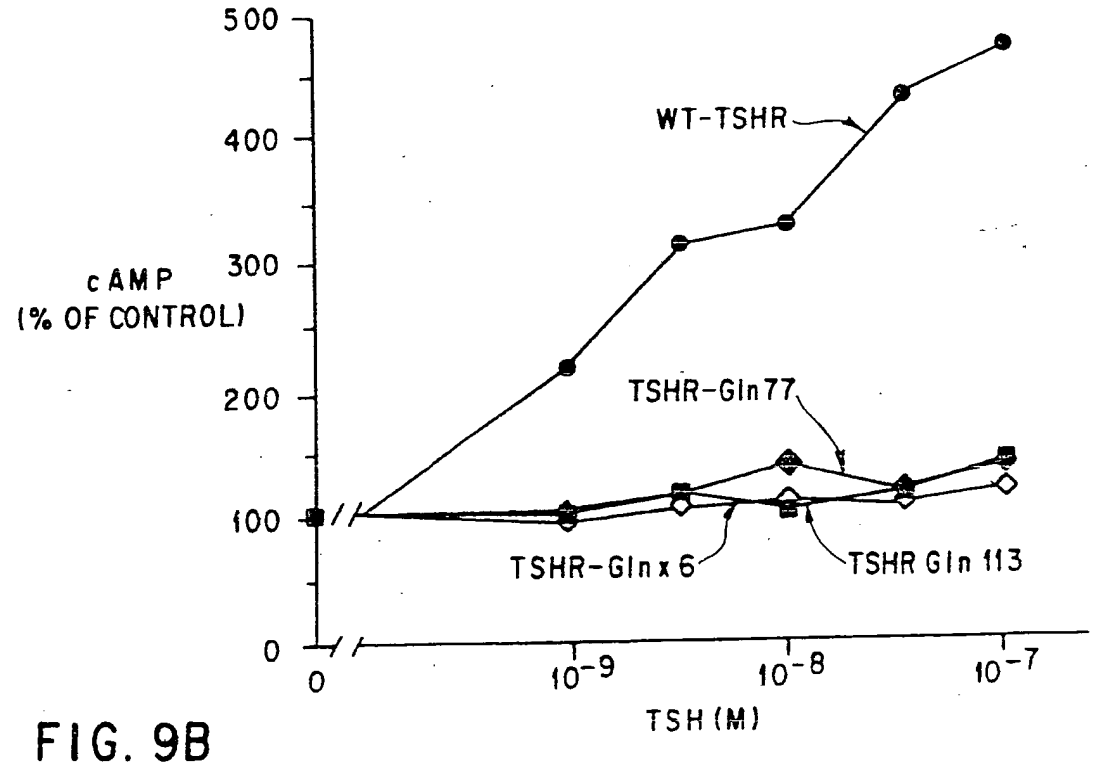

500
400
300
200
100
0
cAMP
(% OF CONTROL)
WT-TSHR
TSHR-Gln77
TSHR-Glnx6
TSHR Gln 113
0
10-9
10-8
10-7
TSH (M)

FIG. 9B

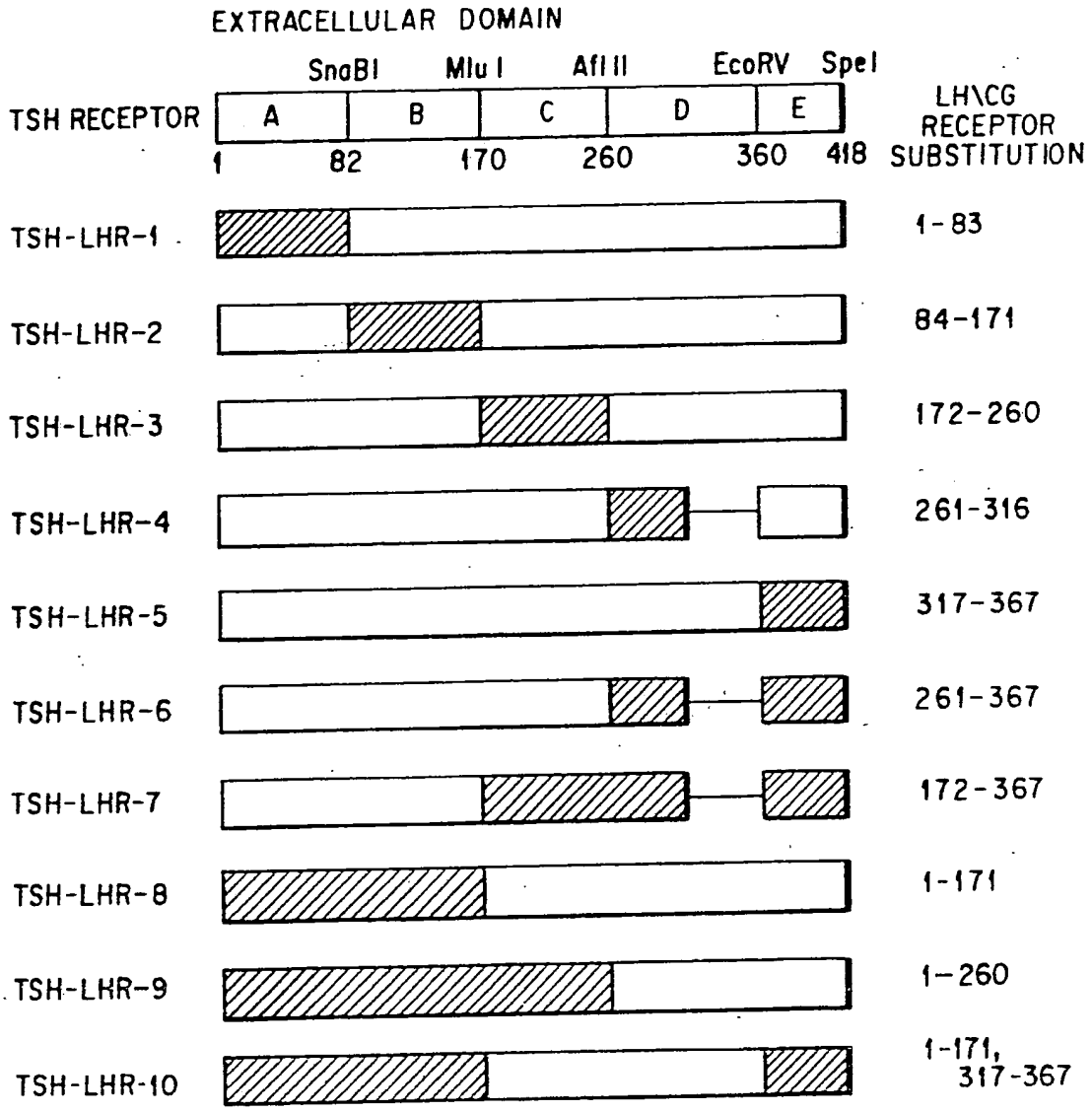

EXTRACELLULAR DOMAIN
SnaBI
Mlu I
Afl II
EcoRV
Spe I
TSH RECEPTOR
A
B
C
D
E
1
82
170
260
360
418
LH\CG RECEPTOR SUBSTITUTION
TSH-LHR-1
1-83
TSH-LHR-2
84-171
TSH-LHR-3
172-260
TSH-LHR-4
261-316
TSH-LHR-5
317-367
TSH-LHR-6
261-367
TSH-LHR-7
172-367
TSH-LHR-8
1-171
TSH-LHR-9
1-260
TSH-LHR-10
1-171, 317-367

FIG.10

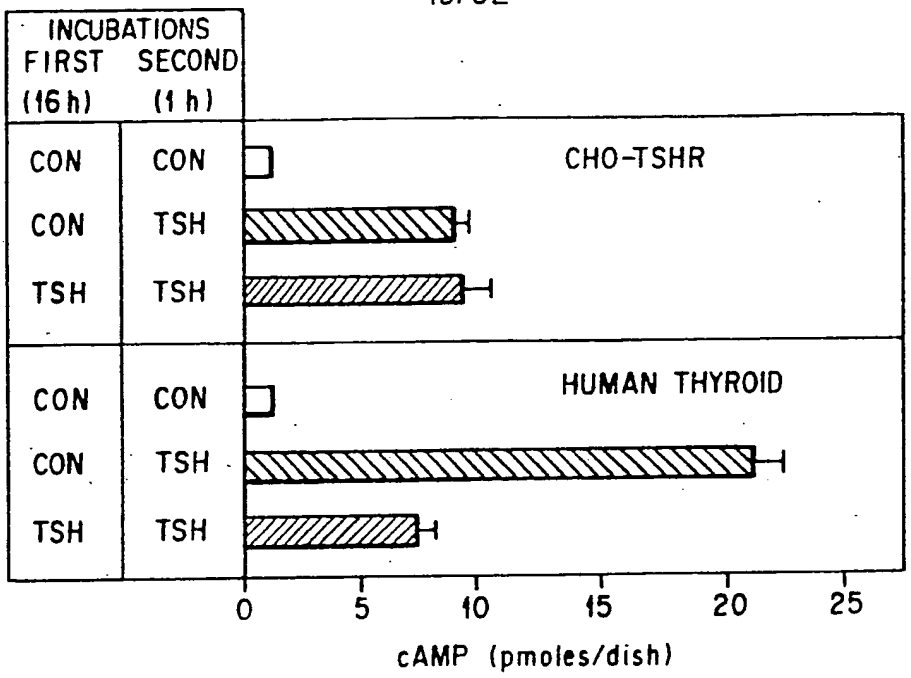

INCUBATIONS
FIRST (16 h)
SECOND (1 h)
CON CON
CON TSH
TSH TSH
CHO-TSHR
CON CON
CON TSH
TSH TSH
HUMAN THYROID
0 5 10 15 20 25
cAMP (pmoles/dish)

FIG. 11

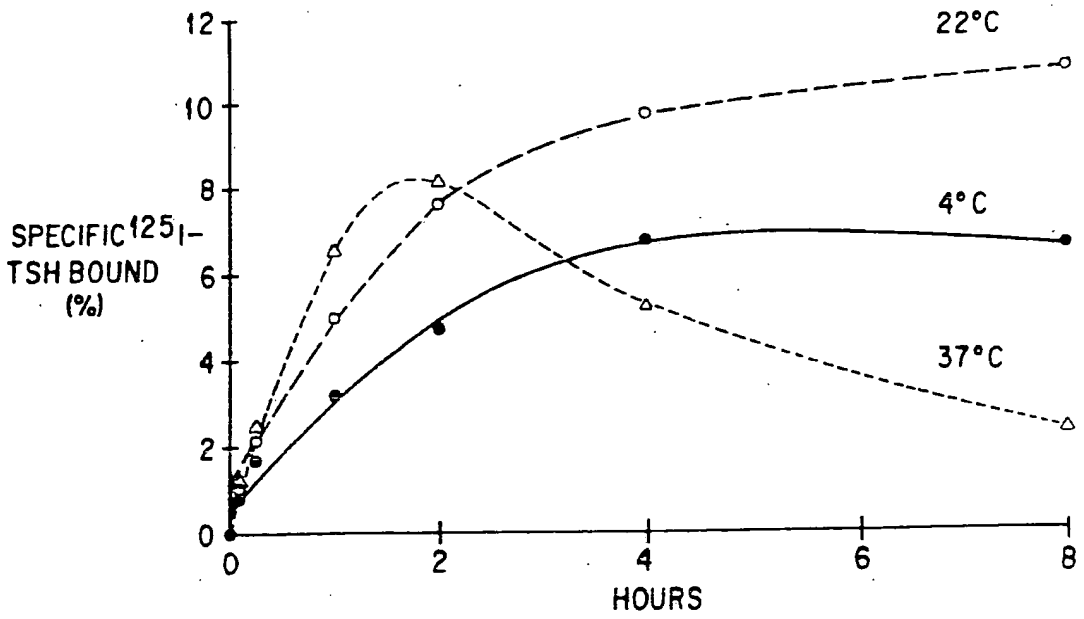

SPECIFIC 125I-TSH BOUND (%)
0 2 4 6 8 10 12
0 2 4 6 8
HOURS
22°C
4°C
37°C

FIG. 12

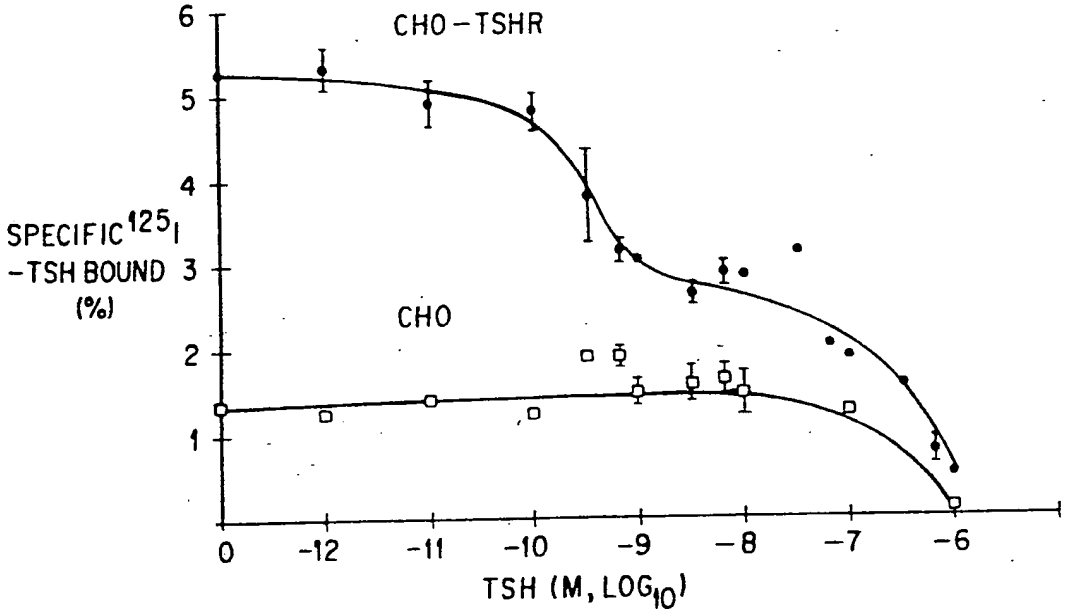

CHO-TSHR
CHO
SPECIFIC ^{125}I -TSH BOUND (%)
TSH (M, LOG$_{10}$)
6
5
4
3
2
1
0
-12
-11
-10
-9
-8
-7
-6

FIG. 13

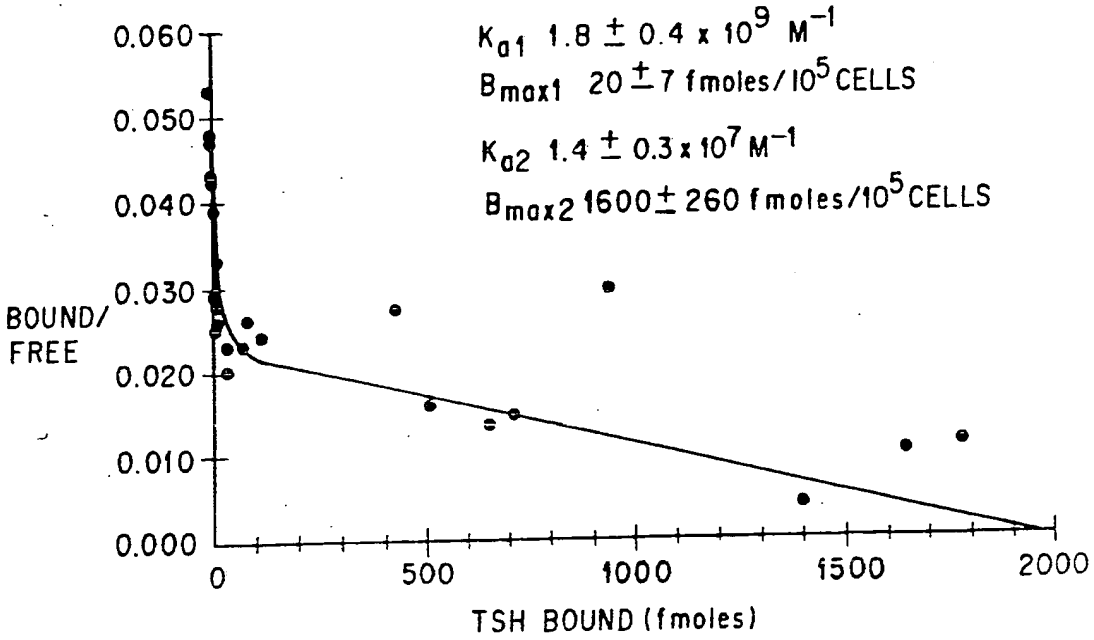

K_{a1} 1.8 ± 0.4 x 10^9 M^{-1}
B_{max1} 20 ± 7 fmoles/10^5 CELLS
K_{a2} 1.4 ± 0.3 x 10^7 M^{-1}
B_{max2} 1600 ± 260 fmoles/10^5 CELLS
BOUND/ FREE
TSH BOUND (fmoles)
0.060
0.050
0.040
0.030
0.020
0.010
0.000
0
500
1000
1500
2000

FIG. 14

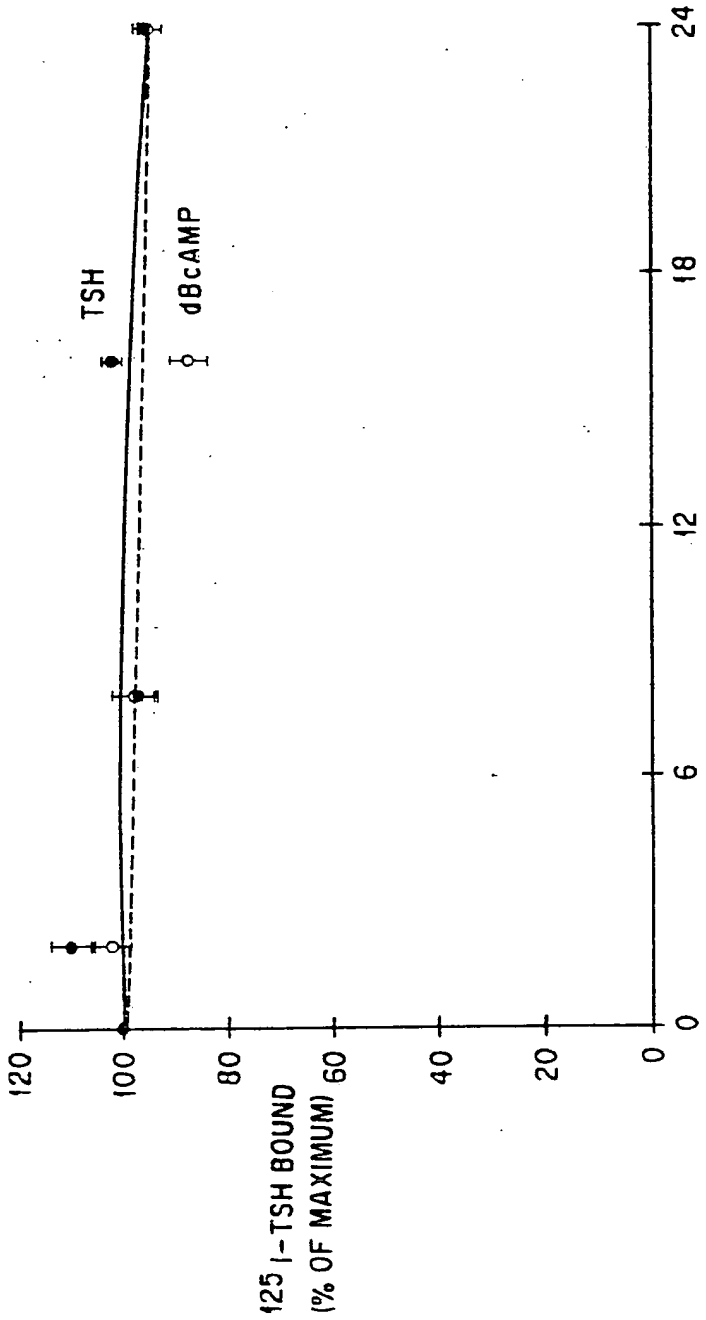

TSH
dBcAMP
120
100
80
60
40
20
0
^{125}I-TSH BOUND
(% OF MAXIMUM)
0
6
12
18
24
HOURS

FIG. 15

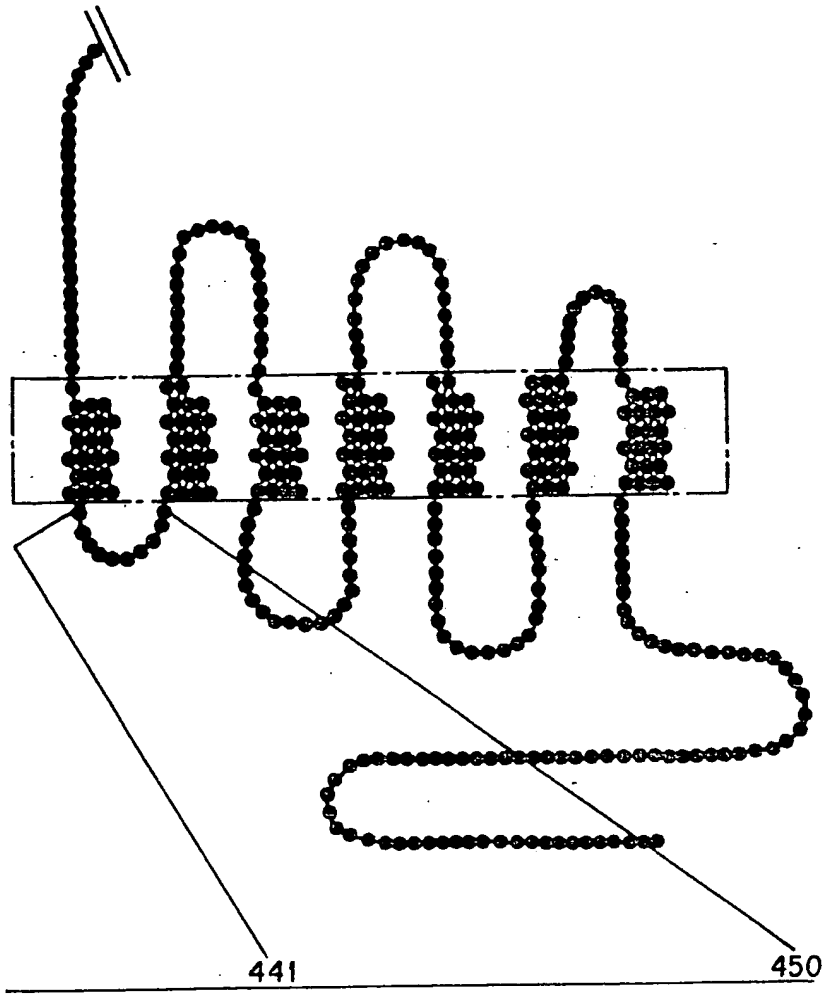


FIG. 16A

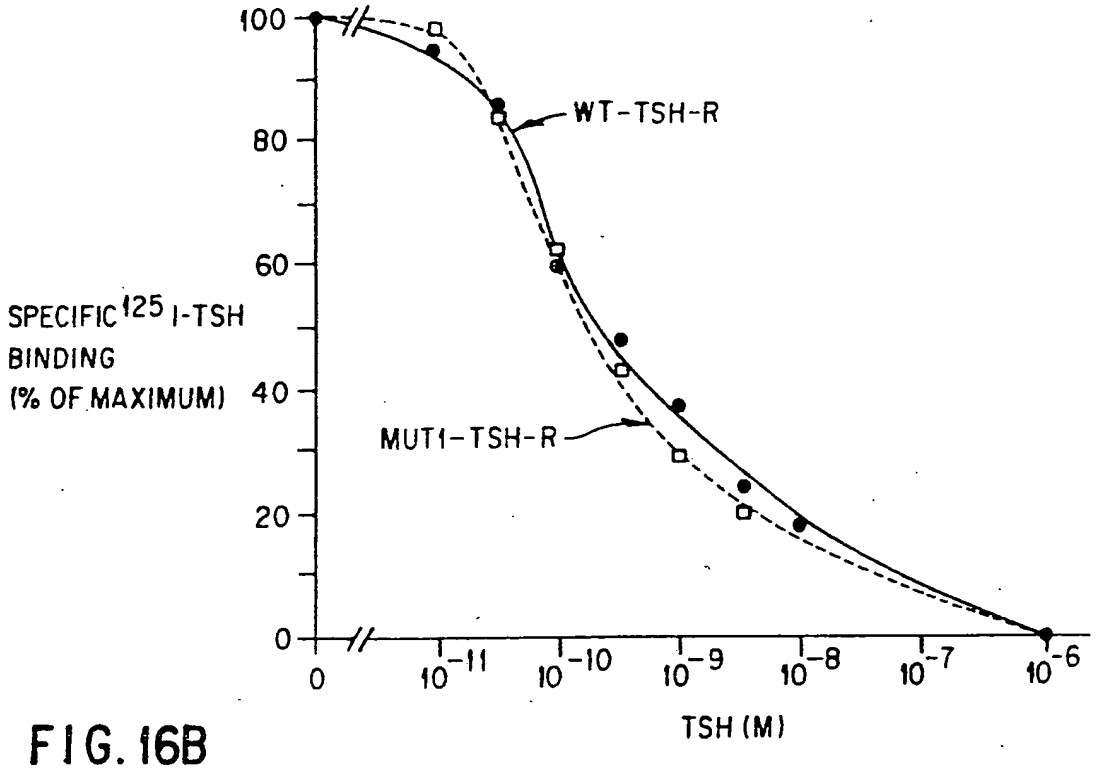


FIG. 16B

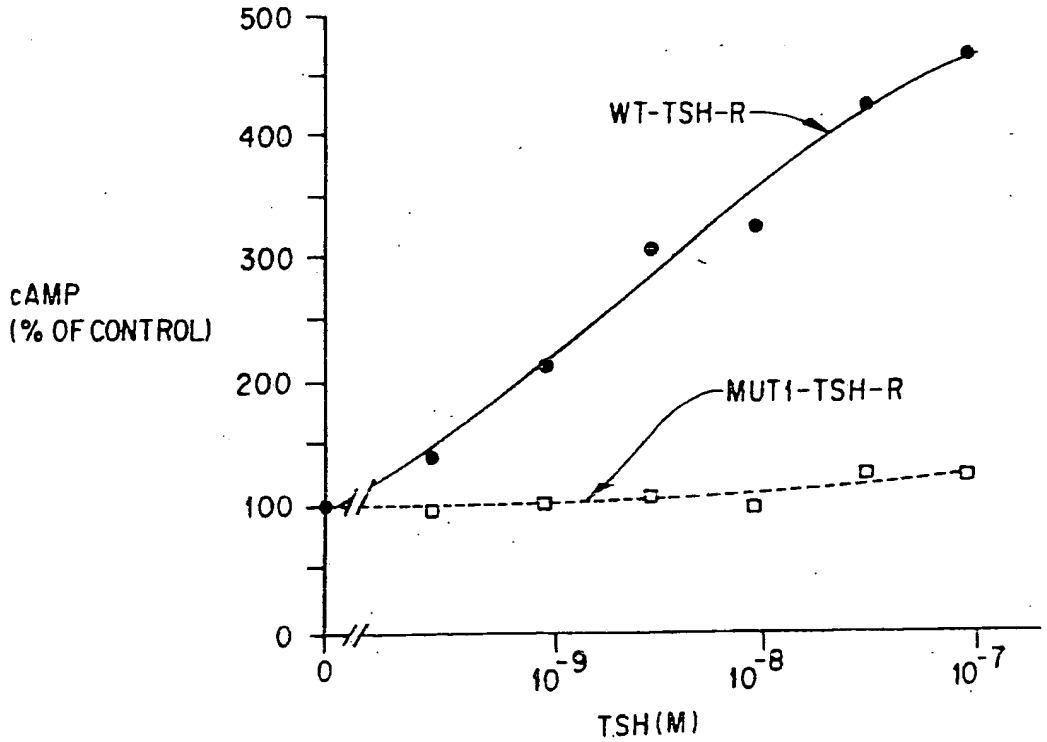
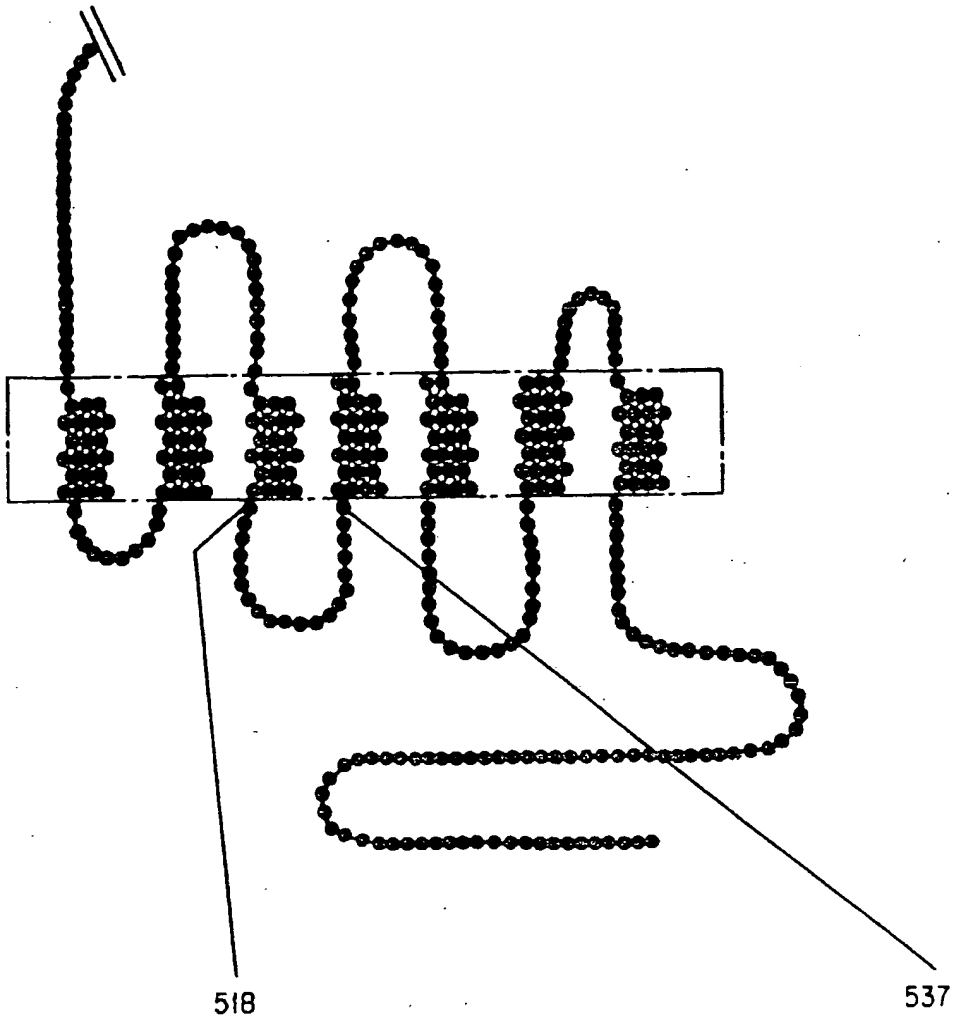


FIG. 16C



WT-TSH-R	E	R	W	Y	A	I	T	F	A	M	R	L	D	R	K	I	R	L	R	H
MUT2-TSH-R	E	R	W	Y	A	I	T	F	A	M	Q	L	G	Q	Q	I	G	L	Q	G
MUT3-TSH-R	Q	G	W	A	A	I	A	F	A	M	R	L	D	R	K	I	R	L	R	H

FIG. 17A

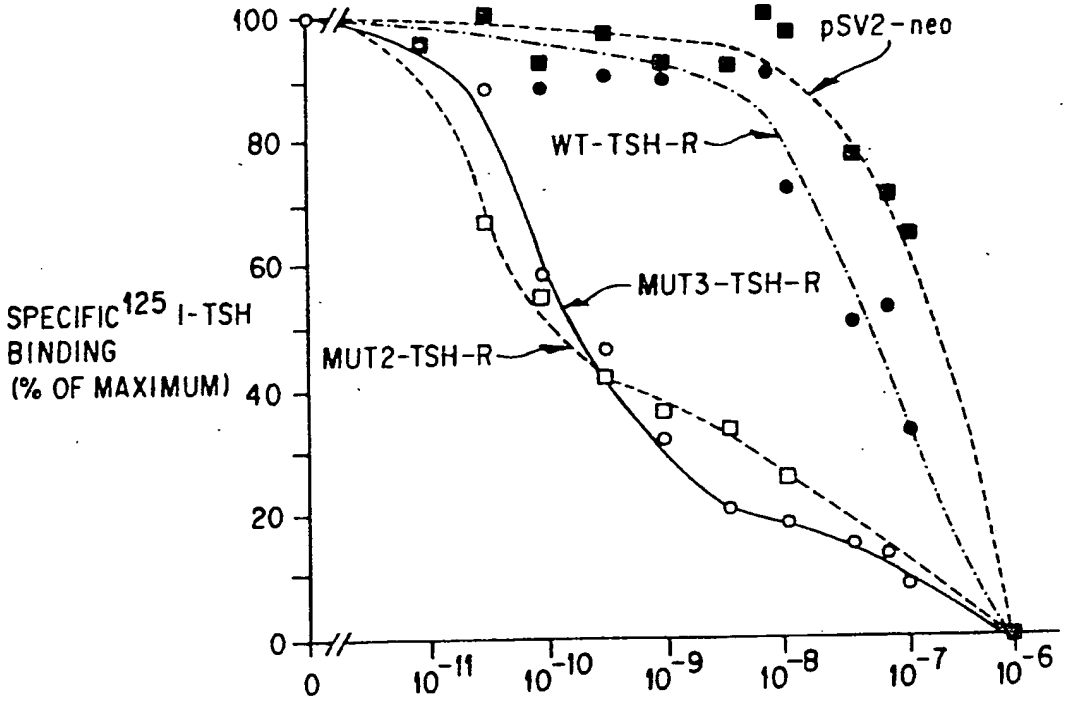


FIG. 17B

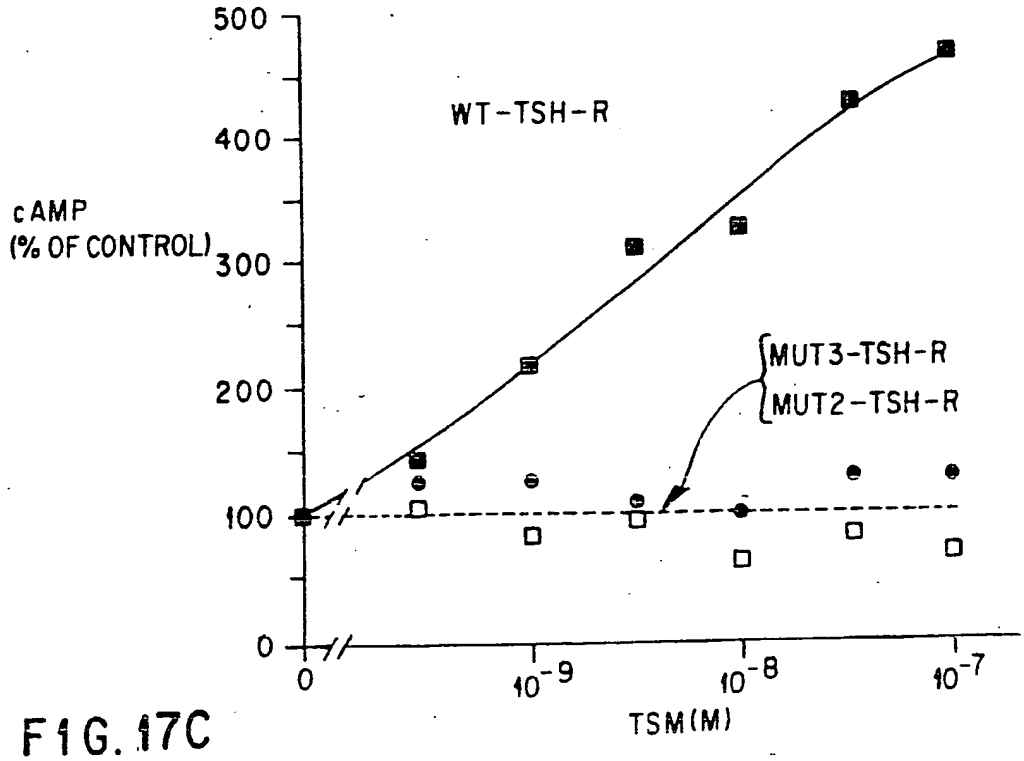
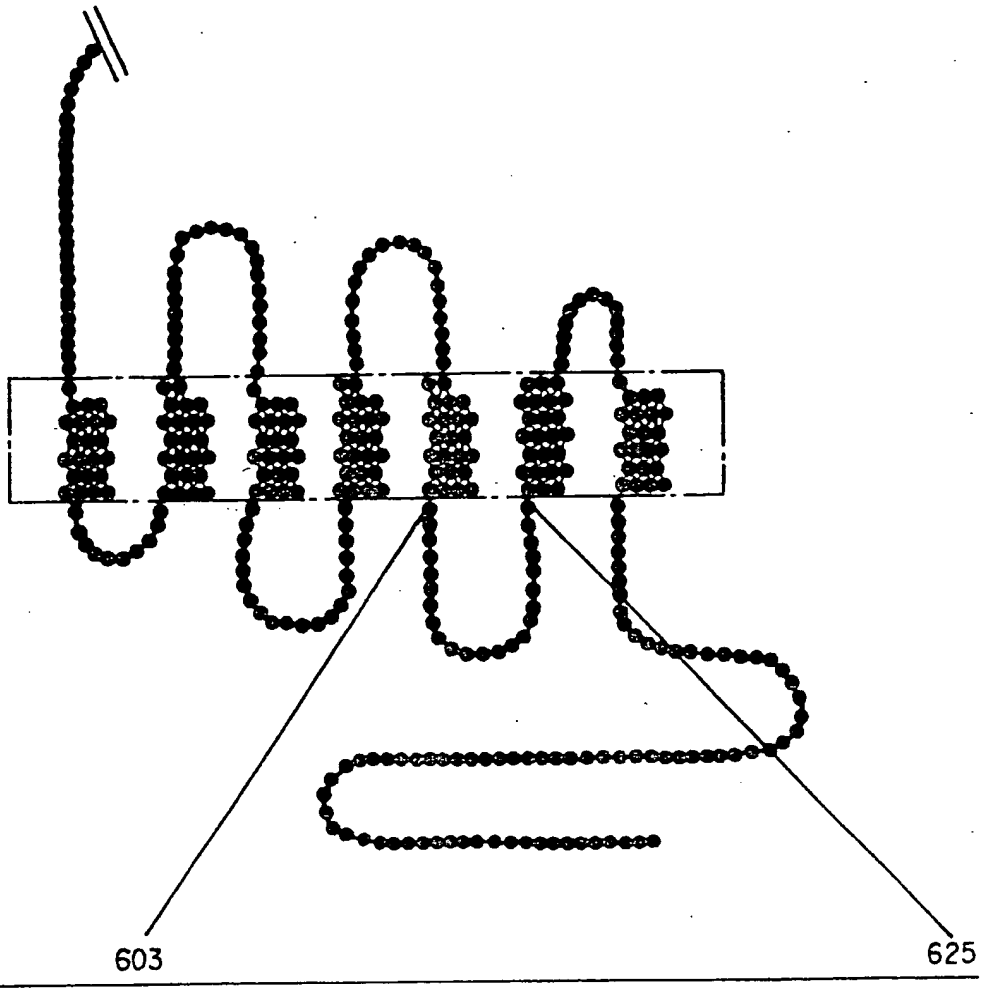


FIG. 17C



WT-TSH-R	K	I	Y	I	T	V	R	N	P	Q	Y	N	P	G	D	K	D	T	K	I	A	K	R
MUT4-TSH-R	K	I	Y	I	T	V	R	N	P	Q	Y	N	P	G	**N**	**Q**	**N**	**A**	**G**	I	A	**Q**	**G**
MUT5-TSH-R	**G**	I	**A**	I	**A**	V	**G**	N	P	Q	**A**	N	P	G	D	K	D	T	K	I	A	K	R

FIG.18A

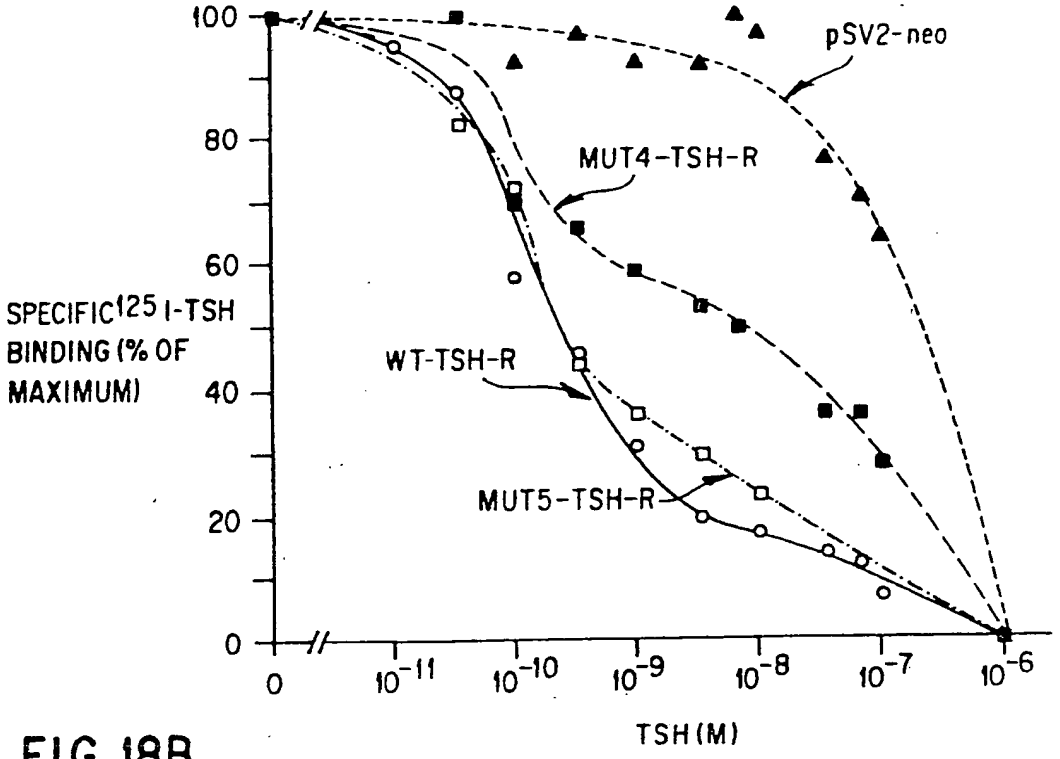


FIG. 18B

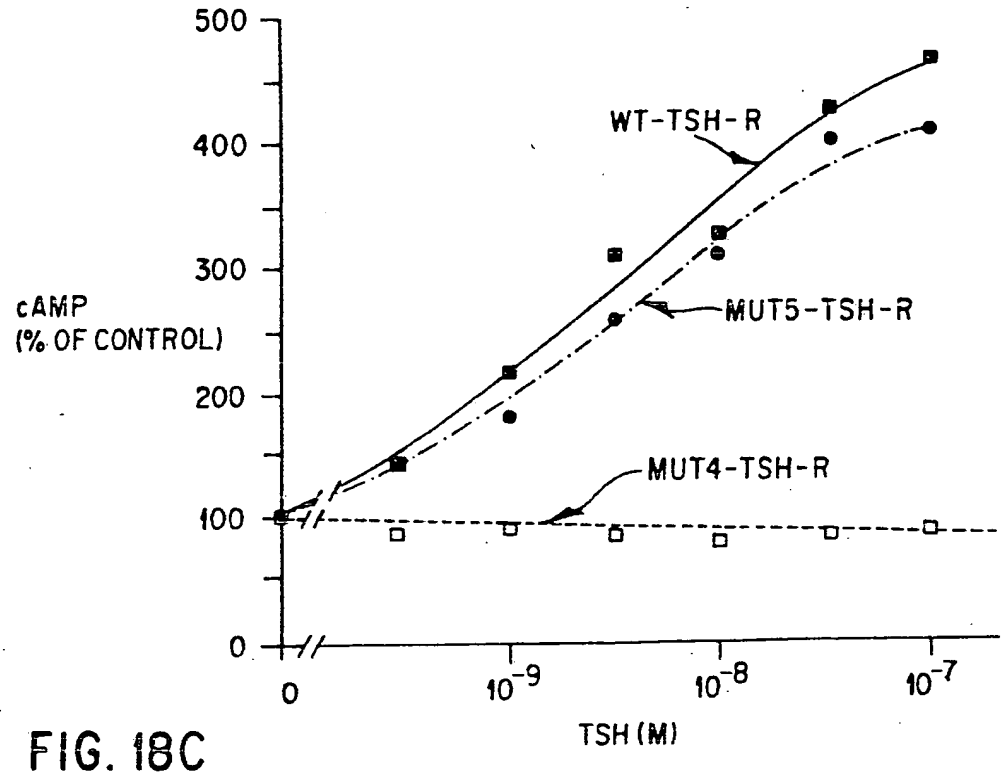


FIG. 18C

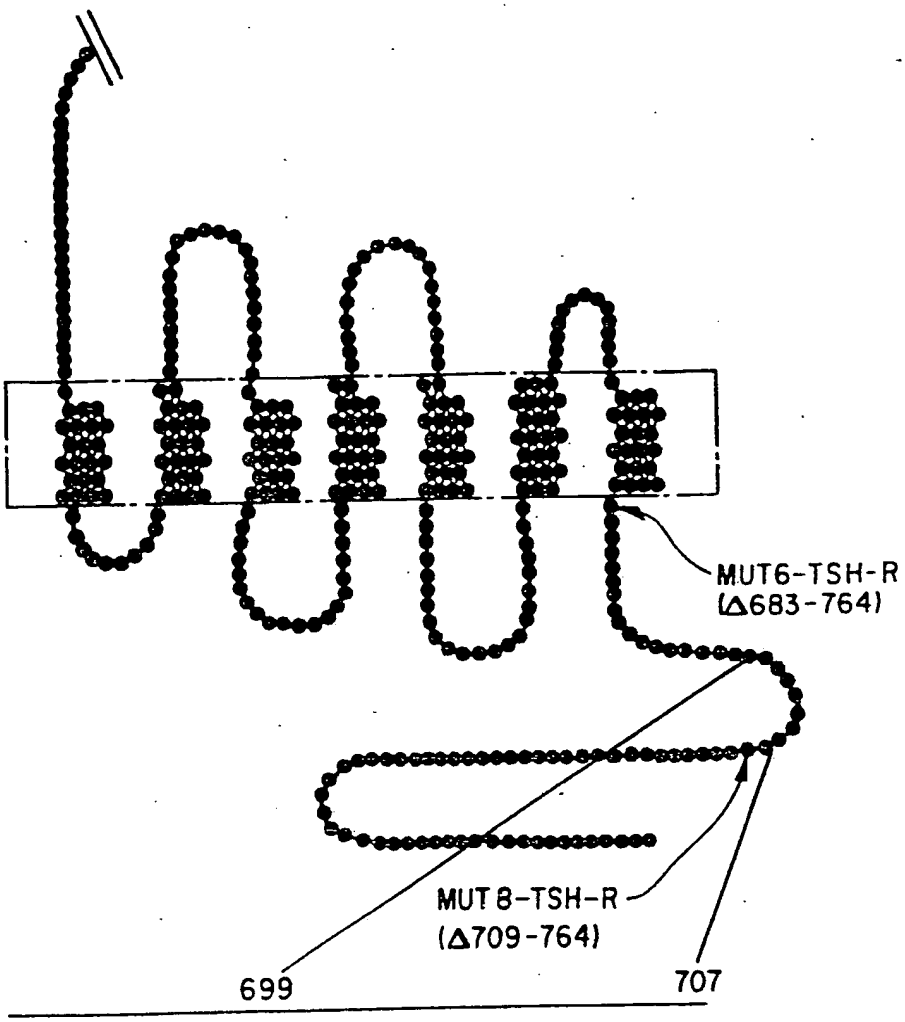

MUT6-TSH-R
(Δ683-764)
MUT 8-TSH-R
(Δ709-764)
699
707
WT-TSH-R C K R Q A Q A Y R
MUT7-TSH-R G Q G Q A Q Y F Q

FIG. 19A

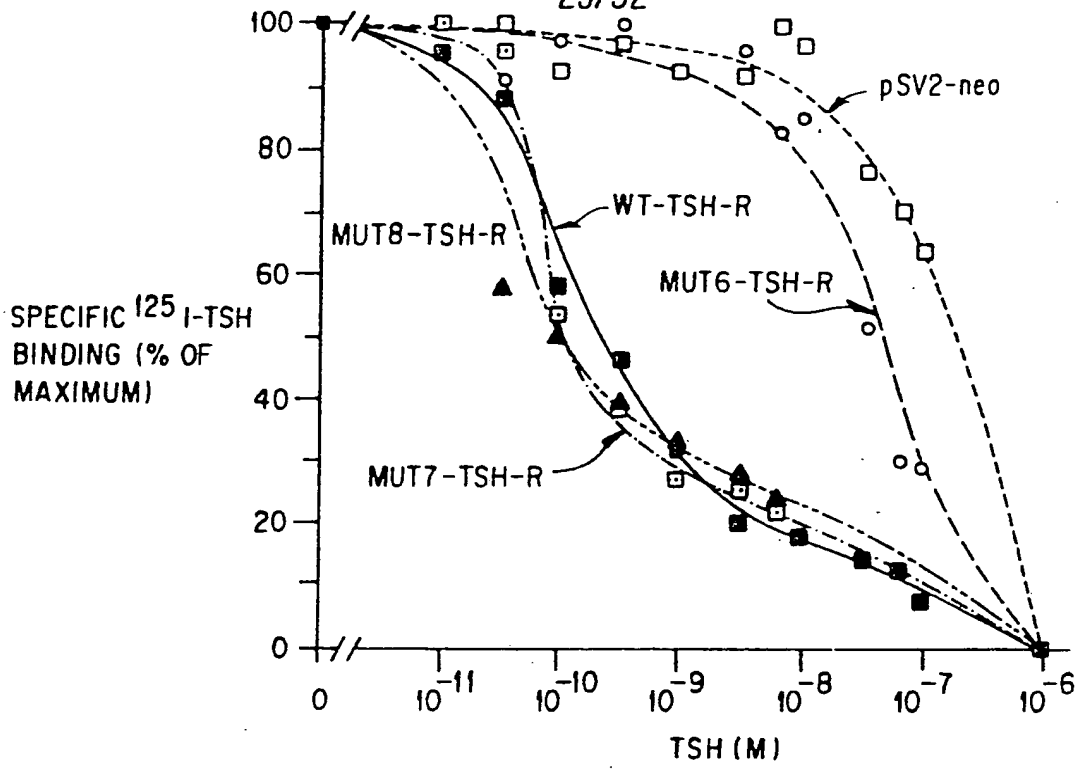


FIG. 19B

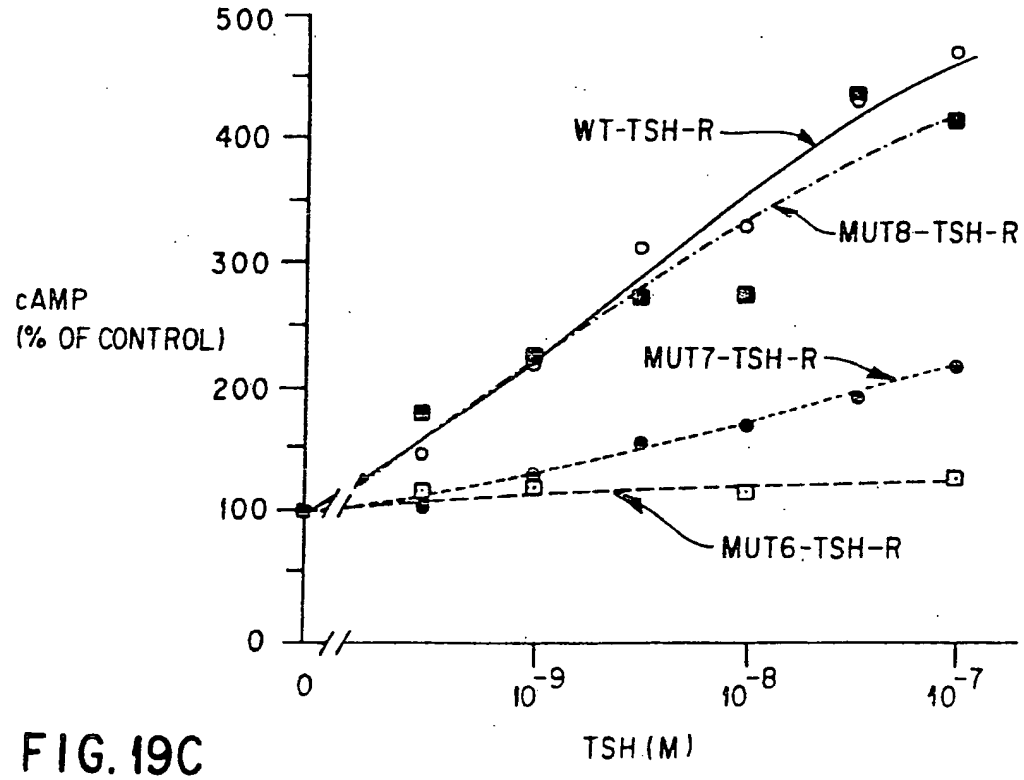


FIG. 19C

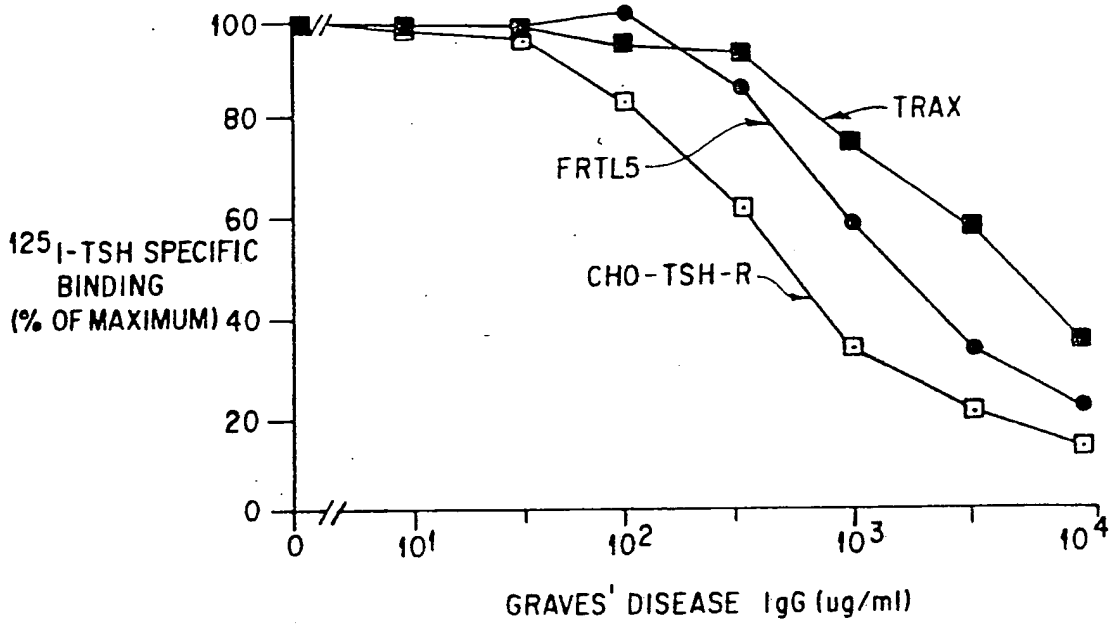

100
80
60
40
20
0
^{125}I-TSH SPECIFIC BINDING (% OF MAXIMUM)
TRAX
FRTL5
CHO-TSH-R
0
10^1
10^2
10^3
10^4
GRAVES' DISEASE IgG (ug/ml)

FIG. 20

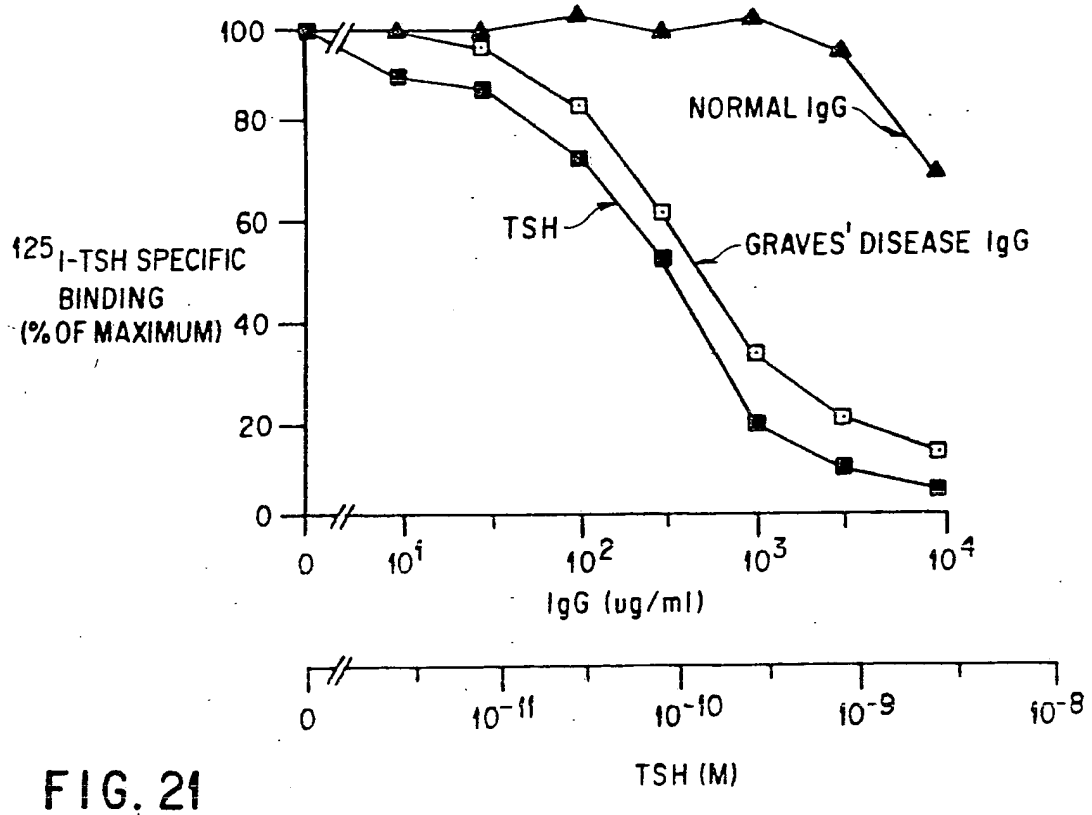

100
80
60
40
20
0
^{125}I-TSH SPECIFIC BINDING (% OF MAXIMUM)
NORMAL IgG
TSH
GRAVES' DISEASE IgG
0
10^1
10^2
10^3
10^4
IgG (ug/ml)
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
TSH (M)

FIG. 21

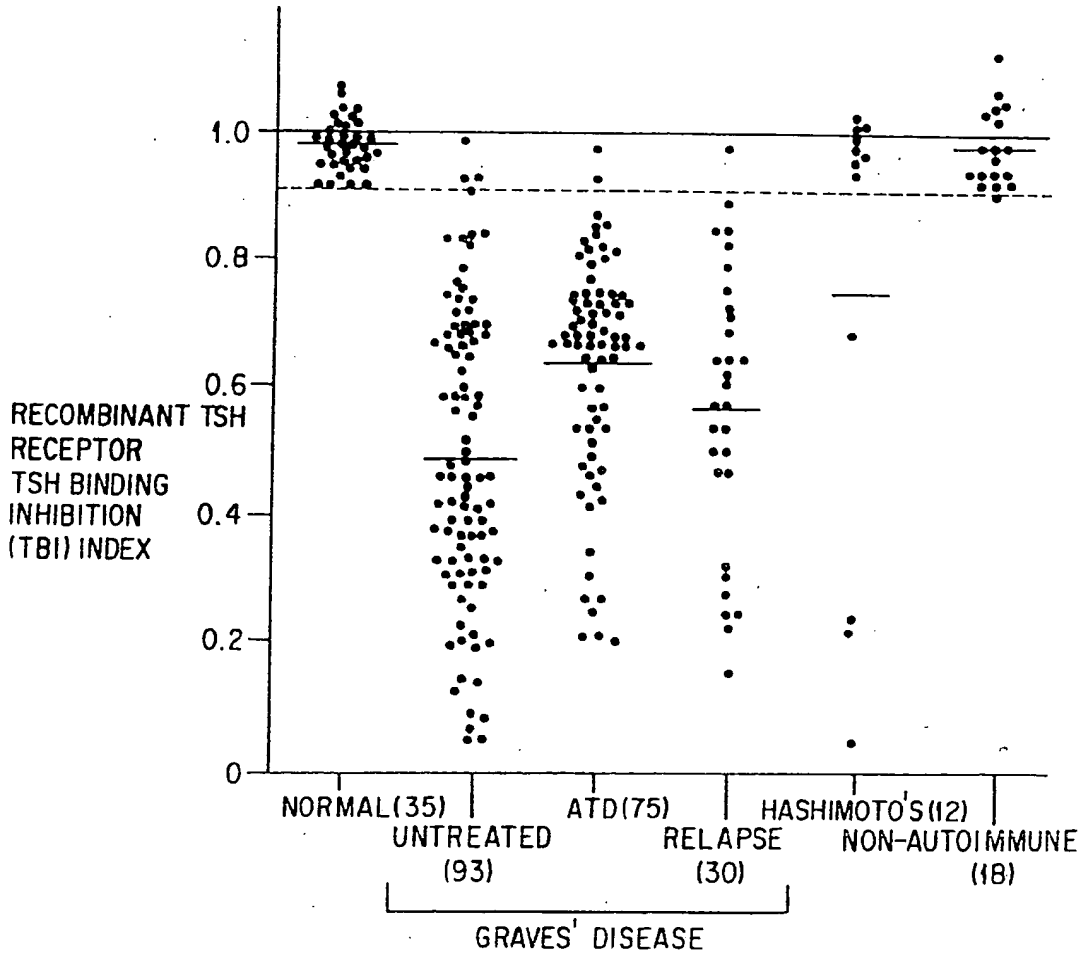

RECOMBINANT TSH RECEPTOR TSH BINDING INHIBITION (TBI) INDEX
1.0
0.8
0.6
0.4
0.2
0
NORMAL(35)
UNTREATED (93)
ATD(75)
RELAPSE (30)
HASHIMOTO'S(12) NON-AUTOIMMUNE (18)
GRAVES' DISEASE

FIG. 22

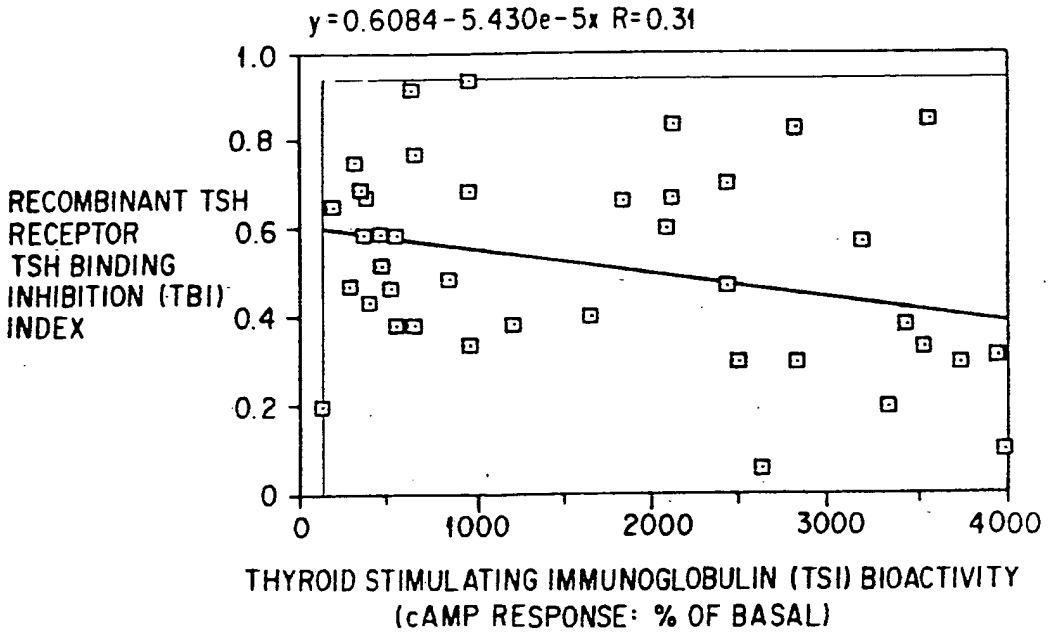

y = 0.6084 - 5.430e-5x R=0.31
RECOMBINANT TSH RECEPTOR TSH BINDING INHIBITION (TBI) INDEX
1.0
0.8
0.6
0.4
0.2
0
0
1000
2000
3000
4000
THYROID STIMULATING IMMUNOGLOBULIN (TSI) BIOACTIVITY
(cAMP RESPONSE: % OF BASAL)

FIG. 23

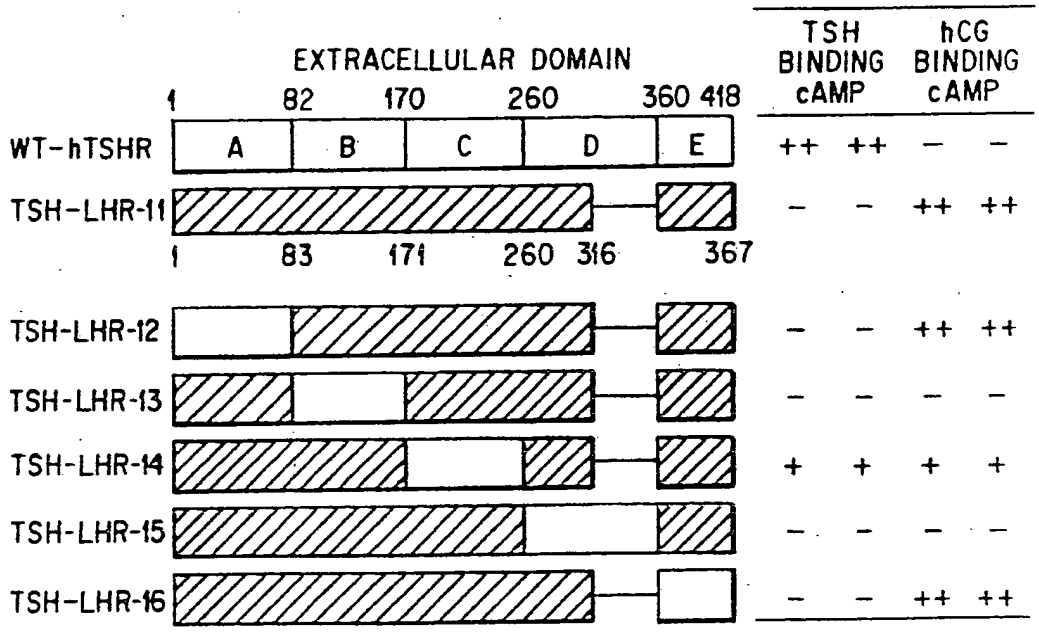

EXTRACELLULAR DOMAIN
1
82
170
260
360 418
WT-hTSHR
A
B
C
D
E
TSH-LHR-11
1
83
171
260 316
367
TSH-LHR-12
TSH-LHR-13
TSH-LHR-14
TSH-LHR-15
TSH-LHR-16
TSH BINDING cAMP
hCG BINDING cAMP
++ ++ – –
– – ++ ++
– – ++ ++
– – – –
+ + + +
– – – –
– – ++ ++

FIG. 24

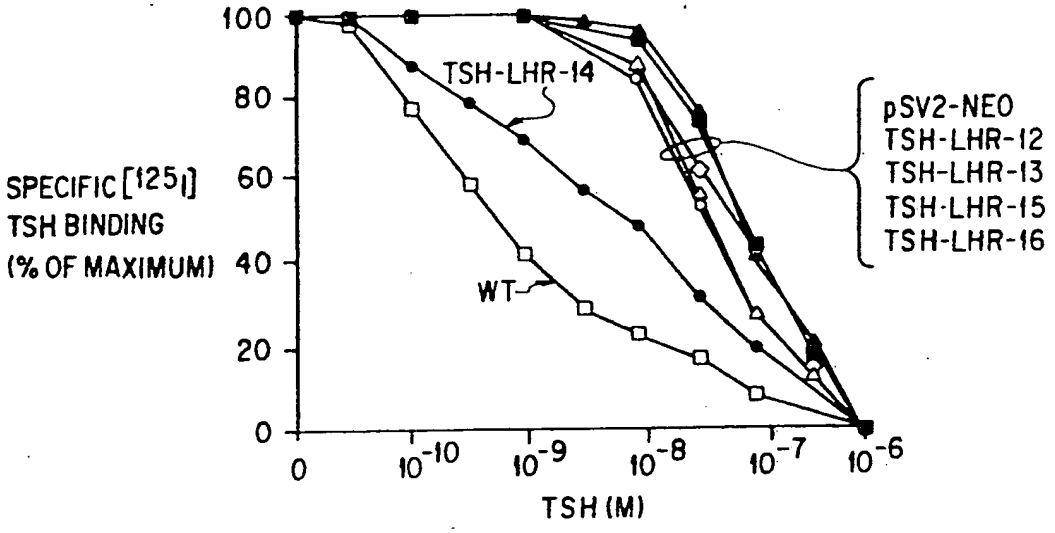


FIG. 25A

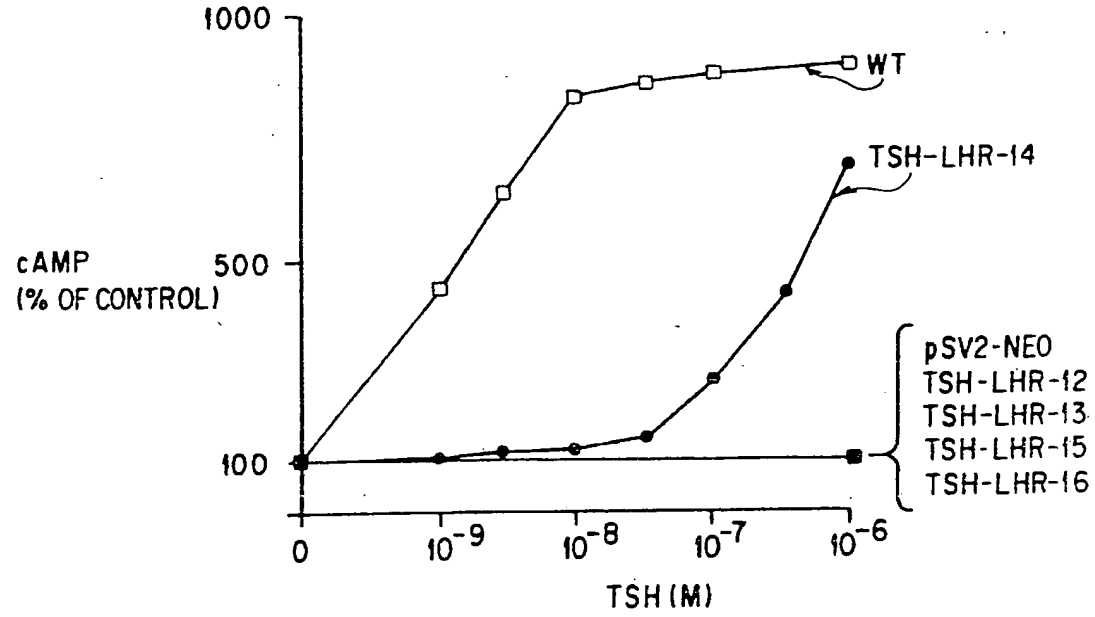


FIG. 25B

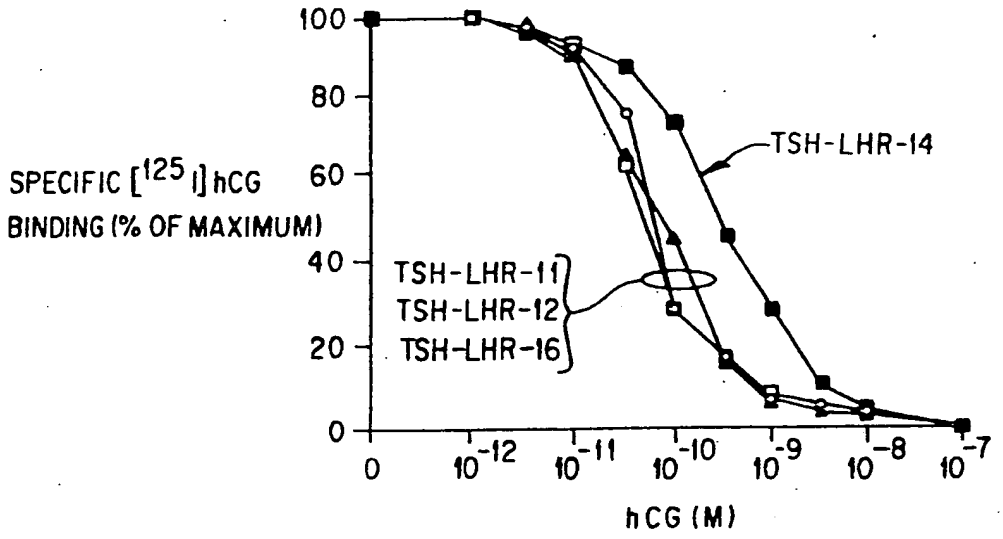


FIG. 26A

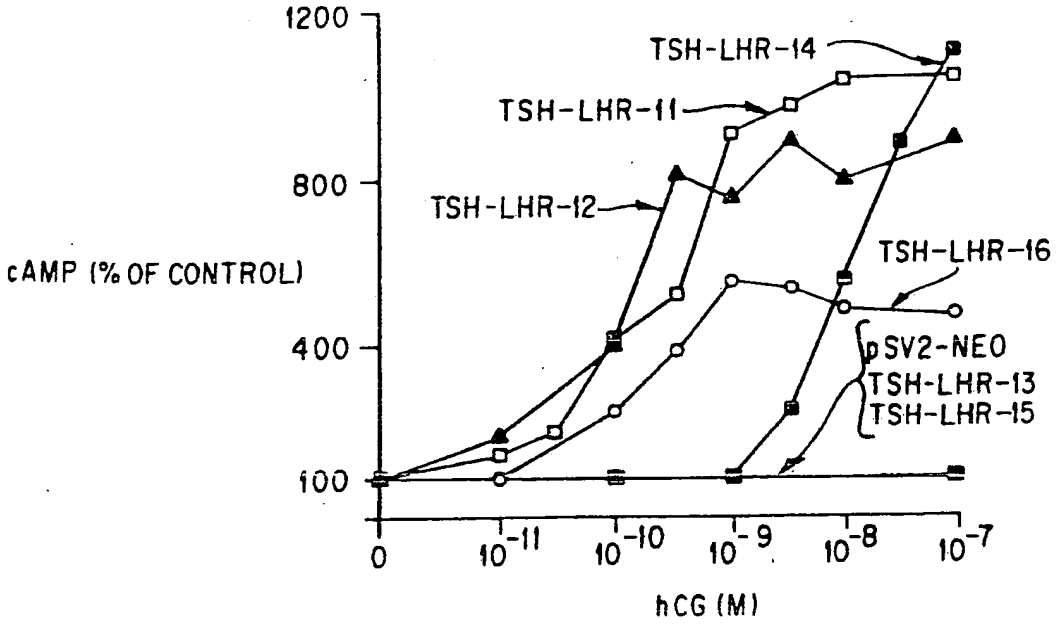


FIG. 26B

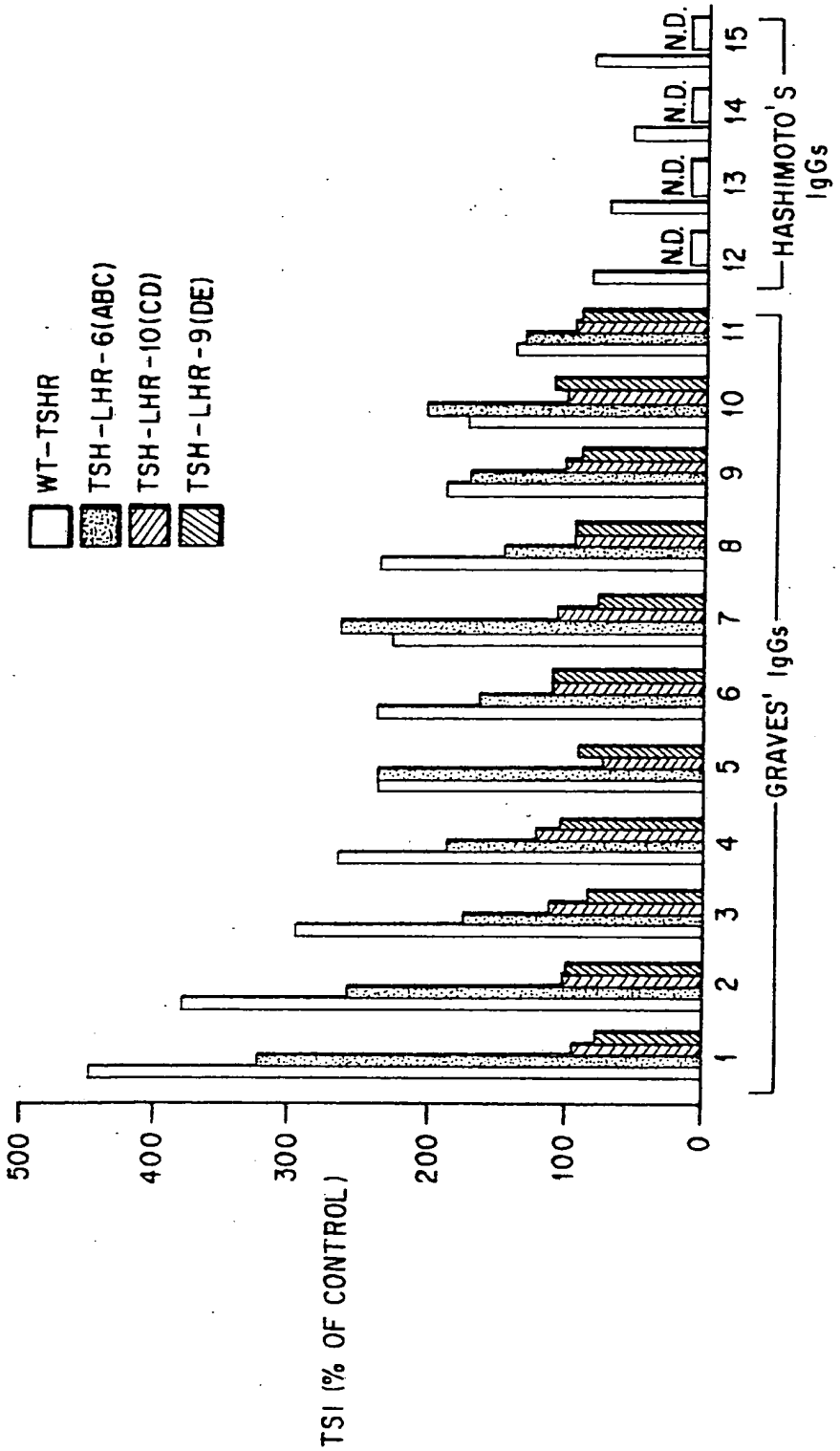

WT-TSHR
TSH-LHR-6(ABC)
TSH-LHR-10(CD)
TSH-LHR-9(DE)
TSI (% OF CONTROL)
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
N.D.
N.D.
N.D.
N.D.
GRAVES' IgGs
HASHIMOTO'S IgGs

FIG. 27

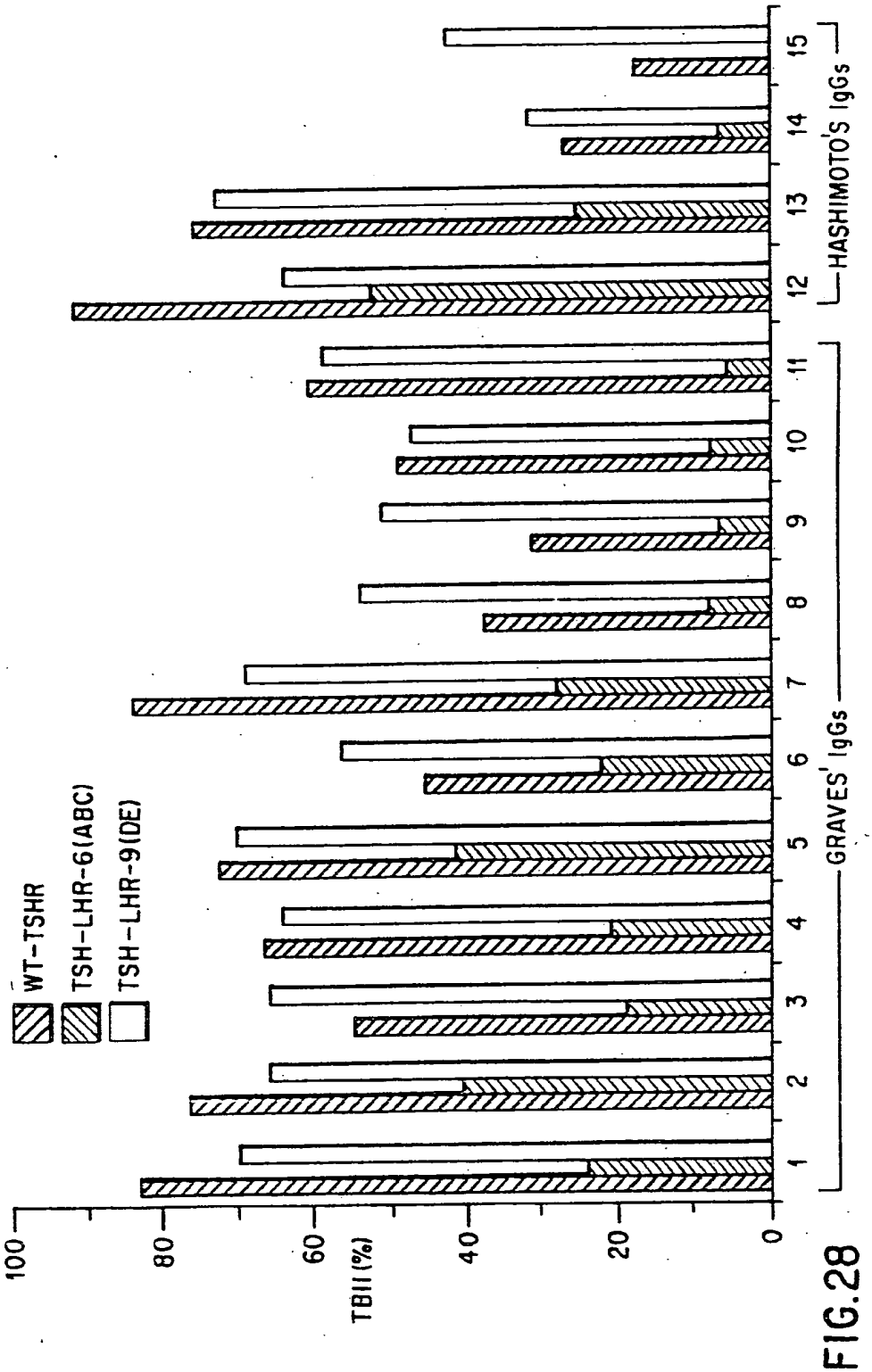

WT-TSHR
TSH-LHR-6(ABC)
TSH-LHR-9(DE)
TBII(%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
GRAVES' IgGs
HASHIMOTO'S IgGs

FIG.28

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/07387

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/00; 1/68; G01N 33/53

U.S. CL: 435/6, 7.21, 172.3, 252.3; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5, 6, 7.1, 7.21, 7.94, 69.3, 172.3, 252.3, 320.1 436/501, 506; 536/27; 530/808

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Services Online (File CA, 1967-1991; File Biosis Preview 1969-1991). Automated Patent System (File USPAT, 1975-1991).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,818,684 (**EDELMAN ET AL.**) 04 April 1989, See the abstract.	7,8,10,11,13, 14,17, 18, 20-26 & 28
P,Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, volume 165, No. 3, issued 29 December 1989, **NAGAYAMA ET AL.**, "Molecular Cloning, Sequence and Functional Expression of the CDNA For The Human Thyrotropin Receptor", pages 1184-1190, see entire article.	1-6,9,12,15,16 19,27,34-37
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, volume 82, issued January 1985, **T.A. KUNKEL**, "Rapid And Efficient Site-Specific Mutagenesis Without Phenotypic Selection", pages 488-492, See the abstract.	9,10,12,13, 15-17, 19, 34,36
Y	ENDOCRINOLOGY , Volume 122, No. 6, issued 1988, **HILL ET AL.** "Monoclonal Antibodies To The Thyrotropin Receptor Raised By An Autoantidiotypic Protocol And Their Relationship To Monoclonal Autoantibodies From Graves' Patients", pages 2840-2850, See the Abstract.	7,8,10,11,13, 14,17,18, 20-26 & 28

(cont.)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 April 1991	24 MAY 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Laurie A. Scheiner (vsh)

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 263, No. 31, issued 05 November 1988, **YOSHIDA ET AL.**, "Monoclonal Antibodies To The Thyrotropin Receptor Bind to A 56-kDa Subunit Of The Thyrotropin Receptor And Show Heterogeneous Bioactivities", pages 16341-16347, see abstract.	7,8,10,11,13, 14,17,18, 20-26 & 28
A	NATURE, Volume 341, issued 12 October 1989, **VANDENBARK ET AL.** "Immunization With A Synthetic T-cell Receptor V-region peptide Protects Against Experimental Autoimmune Encephalomyelitis", pages 541-544, see abstract.	29-33
A	SCIENCE, Volume 245, issued 04 August 1989, **McFARLAND ET AL.**, "Lutropin-Choriogonadotropin Receptor: An Unusal Member Of The G Protein-coupled Receptor Family", pages 494-499, see abstract.	1-3, 5 & 6